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Analysis of the Histocompatibility-2 Locus in the Mouse [1, 2]

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The histocompatibility-2 (*H-2*) locus of the mouse is a locus determining susceptibility and resistance to tumor (and presumably also normal tissue) transplants. The locus lies on the ninth chromosome in close association with the genes kinky tail (*Ki*), fused tail (*Fu*), and brachyury or short tail (*T*), themselves all closely linked (*1*). Preliminary crossover tests gave 4.1 percent recombination between *H-2* and *T* in heterozygous males, and 12.5 percent in heterozygous females (*2*).

This linkage has made it possible to analyze the alleles at the *H-2* locus (*3*). Five alleles, designated by the symbols $H\text{-}2^{dk}$, $H\text{-}2^{d}$, $H\text{-}2^{k}$, $H\text{-}2^{b}$, and $H\text{-}2^{p}$, have been reported to date (*4*). The alleles $H\text{-}2^{d}$ and $H\text{-}2^{k}$ are of interest in that hybrid mice of the genotype $H\text{-}2^{d}/H\text{-}2^{k}$ will grow strain A ($H\text{-}2^{dk}/H\text{-}2^{dk}$) tumor 15091a (*4*).

The distribution of these five alleles in certain inbred strains has been mapped (*4*), and the two isogenic resistant strains C57BL/6–$H\text{-}2^{d}$ and C57BL/10–$H\text{-}2^{d}$ (*5, 6*) have been shown to be probably the result of mutations from $H\text{-}2^{b}$ to $H\text{-}2^{d}$ (*7*).

The *H-2* locus, besides its effects on transplants, also determines certain red-cell antigens demonstrable through the use of antisera from mice in which foreign-strain tumors have regressed (*8*).

Materials and Methods

The inbred strains of mice used were: A, AKR, BALB/c, CBA, C3H, C57BL/6, C57BL/10, C57BR/a, C57BR/cd, C57L, DBA/1, DBA/2, LP, P, RIII/Wy, ST, YBL/Rr, and 129/Rr. Some of the animals were bred in our own colony, others were kindly contributed by the Inbred Nucleus of the Roscoe B. Jackson Memorial Laboratory or by various persons on

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[4] The authors are greatly indebted to Mrs. Sally Lyman Allen for technical assistance during the early stages of this investigation, and for permission to include, in our table 2, data from several tests made by her at the University of Chicago.

the Laboratory staff. Thus more than one substrain was used. Certain substrains, *e.g.* A/L, C57BL/6Ks, RIII/Jax, were found unsuitable in that they failed to give 100 percent growth of our standard tumors that had originated in ancestral or collateral lines. Cases of this sort can no longer be regarded as surprising since the occurrence of several such substrains, presumably arising by mutation at a histocompatibility locus, has already been demonstrated (*5*, *6*, *9*). Such presumed mutant lines were rigorously avoided.

The gene for *Ki*, *Fu* or *T*, used as a marker or tag in each cross, was introduced from one of the following strains:

A–*T:* This is strain A with the gene *T* introduced by repeated backcrossing. The *H-2* allele on the *T* chromosome is $H\text{-}2^{dk}$.

A–$H\text{-}2^{b}T$: Same as A–*T* except that the *H-2* allele associated with *T* is $H\text{-}2^{b}$.

T^{b}: The gene *T* associated with $H\text{-}2^{b}$, as in A–$H\text{-}2^{b}T$, but not on an inbred background.

F^{c}: The gene *Fu* associated with $H\text{-}2^{dk}$ [(*4*)—note that allele $H\text{-}2^{dk}$ was previously called *H-2*]. This stock was kindly provided by Dr. L. C. Dunn.

F^{b}: The gene *Fu* associated with an *H-2* allele known to be not $H\text{-}2^{dk}$ but otherwise unidentified (*4*).

K8: Stock carrying *Ki*. The *H-2* allele on the *Ki* chromosome has previously been identified as $H\text{-}2^{k}$ (*4*).

K9: *Ki* stock closely related to K8 but carrying a different *H-2* allele. K8 and K9 are derived from mice kindly provided by Dr. L. C. Dunn.

The transplantable tumors employed, with basic facts as to their origin and histologic type, are listed in table 1. Further details in regard to these tumors will be found in the survey by Dunham and Stewart (*10*).

Two methods were used for identification of *H-2* alleles:

I. The Linkage Test

The cross used had the form (M × T) × N, where M and N are any two inbred strains and T is one of the strains carrying *T* (or *Fu* or *Ki*). The offspring from this double cross were inoculated with a tumor native to the strain in the M position.

If $H\text{-}2^{m}$ is the *H-2* allele carried by strain M, and if + stands for the normal allele of gene *T*, then the final cross can have any one of five forms, as follows:

1) $H\text{-}2^{m}+/H\text{-}2^{m}T \times H\text{-}2^{m}+/H\text{-}2^{m}+$, where strains T and N both have the same *H-2* allele as strain M. Since all offspring from this cross carry allele $H\text{-}2^{m}$, characteristic of the tumor donor, they are all susceptible so far as the *H-2* locus is concerned. Hence, barring a second histocompatibility locus on this same chromosome, there will be no evidence of linkage between the *T* locus and susceptibility. If other histocompatibility genes are segregating there may be some resistant animals, but they will be equally distributed among the normal-tail (+) and short-tail (*T*) classes.

TABLE 1.—*Tumors employed and some data as to their behavior*

Tumor	Strain of origin	Date of origin		Histologic type	Results from crosses				
		Spontaneous	Induced		Recombination percent, total all crosses*	Maximum size recorded for tumors that regressed†	Percent regressions among surviving mice	Median survival time (*days*)	Fraction of crosses giving percent + >37.5
S637	P		1948	Fibrosarcoma	12. 1	7. 5	67	38	10/12
C954	C57L	1940		Hepatoma	13. 4	2	28	43	3/5
P1534	DBA/2	1940		Lymphatic leukemia	13. 6	4	55	30	5/6
15091a	A	1928		Anaplastic carcinoma	14. 0	3	34	32	12/12
S653	C57BL/10		1949	Fibrosarcoma ‡	14. 3	0. 8	34	49	7/7
S621	BALB/c		1948	Fibrosarcoma	14. 5	5	73	38	6/8
C1498	C57BL/6	1941		Myeloid leukemia	16. 7	4	63	28	8/9
dbrB	DBA/1	1918		Adenocarcinoma	20. 0	1. 5	13	49	9/12
D1905	C57BR/cd	1944		Adenocarcinoma	26. 1	0. 3	7	50	2/3
S636	C57BR/a		1948	Fibrosarcoma	26. 8	3	68	47	8/8
S629	ST		1948	Fibrosarcoma	28. 3	0. 5	33	40	1/1
S674	RIII/Wy	1950		Carcinoma	32. 9	1	3	67	1/3
S620	CBA		1948	Fibrosarcoma	37. 1	0	0	75	4/4
D22	LP	1949		Carcinoma	47. 3	0. 5	14	40	1/4
6C3HED	C3H		1941	Lymphosarcoma		4	31	29	2/4
Wagner	AKR	1947		Osteogenic sarcoma		1	22	46	1/3

*Excluding *a*) crosses not segregating for a major allelic difference at the *H-2* locus, and *b*) crosses in which fewer than 37.5 percent of the mice were + (succumbed to the tumor).

†Each entry in this column represents, for any given tumor, the largest single observed growth of that tumor that ultimately regressed. The figures are the approximate area in sq. cm. of a section parallel to the body surface. The areas were estimated by eye, and are to be regarded as approximations only, but because of the very large range in size do serve to arrange the tumors in a fairly accurate serial order.

‡Originally a myoblastoma.

2) $H\text{-}2^m+/H\text{-}2^zT \times H\text{-}2^m+/H\text{-}2^m+$, where strains M and N have the same *H-2* allele but strain *T* has a different allele. Again all offspring will carry $H\text{-}2^m$, though some will be heterozygous ($H\text{-}2^z/H\text{-}2^m$). However, since susceptibility is dominant, all will be susceptible, at least so far as this one locus is concerned. Hence there can be no evidence of linkage.

If it is known from prior tests (see crosses 4 and 5 below) that strains M and T in a given cross carry different *H-2* alleles, the absence of linkage in this cross provides evidence that we are dealing with a type 2 cross. In other words if strains M and T carry different *H-2* alleles, and no linkage is manifest, then strains M and N probably carry the *same H-2* allele. Possible exceptions are discussed below.

3) $H\text{-}2^m+/H\text{-}2^mT \times H\text{-}2^n+/H\text{-}2^n+$, where strains M and T have the same *H-2* allele, but strain N has a different allele. Again all offspring will carry $H\text{-}2^m$ and there will be no evidence of linkage.

If it is known from prior tests (see crosses 4 and 5 below) that M and N carry different *H-2* alleles, the absence of linkage then provides evidence that M and T carry the *same H-2* allele. Possible exceptions are discussed below.

4) $H\text{-}2^m+/H\text{-}2^nT \times H\text{-}2^n+/H\text{-}2^n+$, where strains T and N carry an allele $H\text{-}2^n$ distinct from the allele in strain M.

5) $H\text{-}2^m+/H\text{-}2^zT \times H\text{-}2^n+/H\text{-}2^n+$, where each of strains M, T, and N carries its own distinct allele.

Crosses 4 and 5 will produce both mice with $H\text{-}2^m$ and mice without $H\text{-}2^m$, the ratio of the two types being 1:1. The former, since they share the gene $H\text{-}2^m$ with the tumor donor, will be genetically susceptible; the latter will be resistant. Except for crossovers, the former will be normal-tailed (+), the latter short-tailed (*T*). Hence linkage between susceptibility and the *T* locus will be manifest. There are, however, influences operative in certain crosses that may in rare instances obscure the linkage, leading to the false conclusion that strains M and T or M and N in the cross in question carry the same allele, when actually they carry different alleles. These influences will be discussed in detail later. Meanwhile we may state the two following theorems:

(*a*) The occurrence of linkage proves that strains T and N carry an *H-2* allele (or alleles) *different* from that in strain M.

(*b*) The absence of linkage establishes a presumption but does not prove beyond question that strains M and T and/or M and N carry the *same H-2* allele.

These are the basic theorems on which the determination of alleles by the linkage method are based.

II. The Component Test

This test consists of crossing two strains and inoculating the F_1 hybrid with strain A tumor 15091a.

All tumor inoculations were made subcutaneously on the right side by trocar. Tumors were palpated once a week, usually beginning 1 to 3 weeks after inoculation. A record of tumor growth was made by making a

drawing of small tumors, and by entering a number based on an arbitrarily selected number system to indicate the size of larger tumors. Mice were killed only after they had been negative for 3 or more weeks. In the final tabulations, mice were scored as positive (+) if they died from progressive tumor growth, as negative (−) if they survived. Many of the mice scored as negative had had tumors which regressed.

The tails of the mice were scored as + or *T* (or *Fu* or *Ki*) at weaning. A few animals with doubtful tails were scored as *T?* or *Fu?*. These were omitted in the final tabulations. Occasionally, mice with normal tails or doubtful tails were tested genetically, and in a few instances proved to be genetically *Fu* or *T* (so-called "normal overlaps").

Results

I. The Linkage Test

Linkage crosses were set up using many different combinations of stocks The results from 101 of the crosses are entered in table 2. (One entry in this table is the sum of two crosses, bringing the total number of entries or cases to 102.) Results from 6 of the crosses (cases 1, 2, 3, 35, 68, 69) have been previously published. A condensed summary of the data together with a summary of certain data previously published (*3*, *7*) are given in table 3.

For convenience of reference, a case number has been assigned to each cross in table 2. This is given in column 1 of the table. Column 2 shows the three stocks used in any given cross. The offspring of the double cross were in every case inoculated with a tumor native to the first of the three stocks.

Columns 3, 4, 5, and 6 show the number of mice in each of the four classes: normal-tail susceptible (+), normal-tail resistant (−), short-tail (*T*, *Fu*, or *Ki*) susceptible, and short-tail resistant. The second and third classes are the crossover classes, hence deficiency in these classes indicates linkage.

Column 7 shows the recombination percent plus or minus its standard error (calculated from the formula $\sqrt{pq/n}$, where $p=½$, $q=½$). We use the term "recombination" percent rather than "crossover" percent because, as we shall point out later, a good many animals falling in these classes are not true crossovers. If the percent of animals dying from the tumors is less than 21, column 7 shows, instead of the recombination percent, the entry "% + <21." The occurrence of so few positives obscures any linkage that may exist, rendering the data of little value for linkage calculations. When the percent dying is greater than 95, the entry is "% + >95."

Column 8 shows the conclusion drawn from each cross. We start by arbitrarily assigning a symbol to the *H-2* allele in one stock, *i.e.* strain A in case 1.

Originally (*3*, *4*) we assigned to the allele in strain A the symbol *H-2*, but for reasons that will be apparent later we have now changed this to

TABLE 2.—*Data showing linkage, or absence of linkage, between short tail (or fused or kinky tail) and resistance. Susceptibility to tumor growth indicated by + (mouse died of tumor), resistance by −. The tumor used in each case was native to the first strain listed under each cross.*

Case No.	Cross	Number of mice				Recombination percent ± S. E.	Allelic group Strain being tested Conclusions
		Normal tail		Short tail			
		+	−	+	−		
							The $H\text{-}2^d$ group
1	(A × F^b) × DBA/2	12	1†	2	11	11.5 ± 9.8	DBA/2 is not $H\text{-}2^{dk}$ is $H\text{-}2^d$
2	(A × F^b) × BALB/c	17	0	0	11	0.0	BALB/c is not $H\text{-}2^{dk}$
3	(DBA/2 × T^b) × BALB/c	10	0	12	0	% + >95	is $H\text{-}2^d$
							$H\text{-}2^{d'}$
4	(A × A-$H\text{-}2^bT$) × YBL/Rr	15	2	1	13 (3*)	9.7 ± 9.0	YBL/Rr is not $H\text{-}2^{dk}$
5	(BALB/c × A-*T*) × YBL/Rr	22	3	18	15	36.2 ± 6.6	is not $H\text{-}2^d$?
6	(DBA/2 × A-*T*) × YBL/Rr	34	11	17	10	38.9 ± 5.9	?
7	Cases 5 + 6	56	14	35	25	37.7 ± 4.4	is not $H\text{-}2^d$ is $H\text{-}2^{d'}$
							The $H\text{-}2^k$ group
8	(A × F^b) × C57BR/a	8	0	1	7 (1*)	6.3 ± 12.5	C57BR/a is not $H\text{-}2^{dk}$
9	(BALB/c × F^b) × C57BR/a	13	0	3	11	11.1 ± 9.6	is not $H\text{-}2^d$
10	(C57BR/a × A-*T*) × YBL/Rr	17	4	3	12	19.4 ± 8.3	is not $H\text{-}2^{d'}$ is $H\text{-}2^k$
11	(A × F^b) × C57BR/cd	7	0	0	6	0.0	C57BR/cd is not $H\text{-}2^{dk}$
12	(BALB/c × F^b) × C57BR/cd	11	2	1	11	12.0 ± 10.0	is not $H\text{-}2^d$
13	(C57BR/cd × A-*T*) × C57BR/a	22	1	14	0	% + >95	is $H\text{-}2^k$
14	(A × K9) × CBA	5	0	0	8	0.0	CBA is not $H\text{-}2^{dk}$
15	(CBA × A-*T*) × DBA/2	17	2	9	5	33.3 ± 8.7	?
16	(CBA × A-*T*) × YBL/Rr	31	5	20	13	36.3 ± 6.0	is not $H\text{-}2^{d'}$?
17	(C57BR/a × A-*T*) × CBA	54	12	54	21	46.8 ± 4.2	is $H\text{-}2^k$

18	(CBA × A-*T*) × C57BR/a	37	0	14	0	% + >95	is *H-2*^{k}
							C3H
19	(C3H × A-*T*) × C57BR/cd	12	40	6	27	54.1 ± 5.4	is *H-2*^{k}
20	(CBA × A-*T*) × C3H	14	0	18	0	% + >95	is *H-2*^{k}
							ST
21	(ST × F^c) × A	17	12	1	16	28.3 ± 7.4	is not *H-2*^{dk}
22	(DBA/2 × T^h) × ST	11	0	2	5	11.1 ± 11.8	is not *H-2*^{d}
23	(BALB/c × F^h) × ST	11	3	1	9	16.7 ± 10.2	is not *H-2*^{d}
24	(C57BR/a × A-*T*) × ST	5	0	12	0	% + > 95	is *H-2*^{k}
25	(C57BR/cd × A-*T*) × ST	9	23	7	17	53.6 ± 6.7	is *H-2*^{k}
•26	(C3H × A-*T*) × ST	16	7	15	4	52.4 ± 7.7	is *H-2*^{k}
							The *H-2*$^{k'}$ group
							AKR
27	(A × F^h) × AKR	8	3 (1†)	0	9 (2*)	15.0 ± 11.2	is not *H-2*^{dk}
28	(DBA/2 × T^h) × AKR	13	5	2	11	22.6 ± 9.0	is not *H-2*^{d}
29	(BALB/c × F^h) × AKR	6	1	1	11	10.5 ± 11.5	is not *H-2*^{d}
30	(C57BR/a × A-*T*) × AKR	49	7	22	23	28.7 ± 5.0	is not *H-2*^{k}
31	(AKR × T^h) × C57BR/a	0	13	1	14	% + < 21	?
32	(AKR × T^h) × CBA	9	8	23	11	60.8 ± 7.0	is *H-2*^{k}
33	(C3H × A-*T*) × AKR	23	19	20	13	52.1 ± 5.8	is *H-2*^{k}
							is *H-2*$^{k'}$
							K8
34	(C57BR/a × K8) × C57BL/6	23	5	10	4	35.7 ± 7.7	?
							The *H-2*^{b} group
							C57BL/6
35	(C57BL/6 × F^c) × A	10	0	1	10	4.8 ± 10.9	is not *H-2*^{dk}
36	(C57BL/6 × F^c) × BALB/c	9	3	0	18 (1*)	10.0 ± 9.1	is not *H-2*^{d}
37	(C57BL/6 × F^c) × C57BR/a	22	1	2	16	7.3 ± 7.8	is not *H-2*^{k}
38	(C57BL/6 × F^c) × ST	14	7	8	16	33.3 ± 7.5	is not *H-2*^{k}?
39	(C57BL/6 × F^c) × AKR	12	3	0	10	12.0 ± 10.0	is not *H-2*$^{k'}$
							is *H-2*^{b}
							C57BL/10
40	(C57BL/10 × A-*T*) × YBL/Rr	29	7	2	14	17.3 ± 6.9	is not *H-2*$^{d'}$
41	(C57BL/10 × A-*T*) × C57BR/cd	12	1	3	16	12.5 ± 8.8	is not *H-2*^{k}
42	(C57BL/10 × A-*T*) × CBA	19	4	3	12	18.4 ± 8.1	is not *H-2*^{k}
43	(AKR × T^h) × C57BL/10	3	15	1	19	% + < 21	?
44	(C57BL/10 × A-*T*) × C57BL/6	19	0	9	0	% + > 95	is *H-2*^{b}
							C57L
45	(A × F^h) × C57L	11	5	0	7	21.7 ± 10.4	is not *H-2*^{dk}
46	(BALB/c × F^h) × C57L	4	0	0	7	0.0	is not *H-2*^{d}
47	(C57BR/cd × A-*T*) × C57L	32	5	13	19	26.1 ± 6.0	is not *H-2*^{k}

*Numbers in parenthesis indicate tested normal overlaps, *i.e.* mice with phenotypically normal tails, but which were proved by genetic test to be genetically short-tail (*T* or *Fu*).

†Tested genetically, not a normal overlap.

TABLE 2.—*Data showing linkage, or absence of linkage, between short tail (or fused or kinky tail) and resistance. Susceptibility to tumor growth indicated by + (mouse died of tumor), resistance by −. The tumor used in each case was native to the first strain listed under each cross.*—Cont.

Case No.	Cross	Number of mice				Recombination percent ± S. E.	Allelic group Strain being tested Conclusions
		Normal tail		Short tail			
		+	−	+	−		
							C57L—*Cont.*
48	(C57L × A-*T*) × C57BR/cd	40	6	3	18	13.4 ± 6.1	is not *H-2*^{k}
49	(C57BL/6 × F^o) × C57L	10	0	15	0	% + >95	is *H-2*^{b}
50	(C57L × A-*T*) × C57BL/10	20	0	10	0	% + >95	is *H-2*^{b}
							LP
51	(A × F^b) × LP	8	1†	1	8	11.1 ± 11.8	is not *H-2*^{dk}
52	(C57BR/a × A-*T*) × LP	31	5	12	14	27.4 ± 6.4	is not *H-2*^{k}
53	(LP × A-*T*) × C57BR/a	19	18	9	11	47.4 ± 6.6	is *H-2*^{k}
54	(LP × A-*T*) × C57BR/cd	6	17	1	29	% + <21	?
55	(C57BL/6 × F^o) × LP	11	5	11	0	59.3 ± 9.6	is *H-2*^{b}
56	(LP × A-*T*) × C57BL/6	7	14	4	5	60.0 ± 9.1	is *H-2*^{b}
57	(C57BL/10 × A-*T*) × LP	18	9	19	2	58.3 ± 7.2	is *H-2*^{b}
58	(C57L × A-*T*) × LP	3	24	10	13	68.0 ± 7.1	is *H-2*^{b}
59	(LP × A-*T*) × C57L	1	11	7	8	66.7 ± 9.6	is *H-2*^{b}
							129/Rr
60	(A × A-*H-2*^{b}*T*) × 129/Rr	17	3	3	20	13.9 ± 7.6	is not *H-2*^{dk}
61	(DBA/2 × A-*T*) × 129/Rr	8	25	5	27	% + <21	?
62	(C57BR/a × A-*T*) × 129/Rr	17	0	1	12	3.3 ± 9.1	is not *H-2*^{k}
63	(C57BL/10 × A-*T*) × 129/Rr	15	8	22	4	61.2 ± 7.1	is *H-2*^{b}
64	(C57L × A-*T*) × 129/Rr	4	17	4	16	% + <21	?
							A-*H-2*^{b}*T*
65	(C57BL/6 × A-*H-2*^{b}*T*) × A	12	6	9	3	50.0 ± 9.1	is *H-2*^{b}
66	(C57BL/6 × A-*H-2*^{b}*T*) × CBA	18	1	23	1	% + > 95	is *H-2*^{b}
67	(C57L × A-*H-2*^{b}*T*) × A	6	8	6	10	46.7 ± 9.1	is *H-2*^{b}
							H-2^p
							P
68	(A × F^b) × P	13	2	1	10	11.5 ± 9.8	is not *H-2*^{dk}
69	(DBA/2 × F^b) × P	35	3	5	33	10.5 ± 5.7	is not *H-2*^{d}
70	(P × F^b) × YBL/Rr	17	1	1	11	6.7 ± 9.1	is not *H-2*$^{d'}$

71	(P × F^c) × C57BR/a	15	3	0	10	10.7 ± 9.5	is not *H-2^k*
72	(P × F^c) × C57BR/cd	20	2	2	11	11.4 ± 8.5	is not *H-2^k*
73	(P × F^c) × CBA	9	2	0	18	6.9 ± 9.3	is not *H-2^k*
74	(P × F^c) × ST	35	2	2	23	6.5 ± 6.4	is not *H-2^k*
75	(P × F^c) × AKR	10	8	1	7	34.6 ± 9.8	?
76	(P × F^c) × C57BL/6	13	3	4	9	24.1 ± 9.3	is not *H-2^b*
77	(P × F^c) × C57L	19	1	3	10	12.1 ± 8.7	is not *H-2^b*
78	(P × F^c) × LP	11	1	1	5	11.1 ± 11.8	is not *H-2^b*
79	(P × A–*H-2^{b}T*) × 129/Rr	8	2	1	17	10.7 ± 9.5	is not *H-2^b* is *H-2^p*
							H-2^q DBA/1
80	(DBA/1 × F^c) × A	14	4	0	11	13.8 ± 9.3	is not *H-2^{dk}*
81	(DBA/1 × F^c) × DBA/2	14	1	1	12	7.1 ± 9.5	is not *H-2^d*
82	(DBA/1 × F^c) × BALB/c	9	5	0	15	17.2 ± 9.3	is not *H-2^d*
83	(BALB/c × F^h) × DBA/1	20	0	7	12	17.9 ± 8.0	is not *H-2^d*
84	(DBA/1 × A–*T*) × YBL/Rr	18	7	8	13	32.6 ± 7.4	is not *H-2$^{d'}$*?
85	(DBA/1 × A–*T*) × C57BR/a	17	3	6	13	23.0 ± 8.0	is not *H-2^k*
86	(DBA/1 × F^c) × C57BR/cd	10	3	2	12	18.5 ± 9.6	is not *H-2^k*
87	(DBA/1 × F^c) × CBA	16	3	0	17	8.3 ± 8.3	is not *H-2^k*
88	(DBA/1 × A–*T*) × ST	11	1	0	18	3.3 ± 9.1	is not *H-2^k*
89	(DBA/1 × A–*T*) × AKR	5	13	0	11	% + <21	?
90	(DBA/1 × F^c) × C57BL/6	25	8	8	14	29.1 ± 6.7	is not *H-2^b*
91	(DBA/1 × A–*T*) × 129/Rr	11	6	4	15	27.8 ± 8.3	is not *H-2^b*
92	(P × F^c) × DBA/1	15	1	0	10	3.8 ± 9.8	is not *H-2^p* is *H-2^q*
							H-2^r RIII/Wy
93	(RIII/Wy × A–*T*) × A/Sn	7	17	1	24	% + <21	?
94	(A × A–*H-2^{b}T*) × RIII/Wy	16	3	6	26	17.6 ± 7.0	is not *H-2^{dk}*
95	(BALB/c × A–*T*) × RIII/Wy	17	1	4	15	13.5 ± 8.2	is not *H-2^d*
96	(RIII/Wy × A–*T*) × YBL/Rr	22	15	7	23	32.8 ± 6.1	is not *H-2$^{d'}$*
97	(C57BR/a × A–*T*) × RIII/Wy	18	10	4	16	29.2 ± 7.2	is not *H-2^k*
98	(RIII/Wy × F^h) × C3H	4	16	0	14	% + <21	?
99	(C3H × A–*T*) × RIII/Wy	8	15	2	21	37.0 ± 7.4	?
100	(C57BL/10 × A–*T*) × RIII/Wy	18	3	0	15	8.3 ± 8.3	is not *H-2^b*
101	(P × A–*T*) × RIII/Wy	15	0	3	18	8.3 ± 8.3	is not *H-2^p*
102	(DBA/1 × A–*T*) × RIII/Wy	17	2	1	19	7.7 ± 8.0	is not *H-2^q* is *H-2^r*

†Tested genetically, not a normal overlap.

TABLE 3.—*Condensed summary of recombination percents obtained in linkage tests**

Allelic group →		*H-2^{dk}*	*H-2^{d}*		*H-2^{k}*					*H-2$^{k'}$*	*H-2^{b}*				*H-2^{p}*	*H-2^{q}*	*H-2^{r}*
Tumor donor →		A	DBA/2	BALB/c	C57BR/a	C57BR/cd	CBA	C3H	ST	AKR	C57BL/6	C57BL/10	C57L	LP	P	DBA/1	RIII/Wy
H-2^{dk}	A	.....	.....	.....	.....	.....	.....	.....	28	.....	5	.....	.....	.....	.....	14	–
H-2^{d}	DBA/2	11	.....	+	.....	.....	33	.....	.....	.....	.....	.....	.....	.....	.....	7	.....
	BALB/c	0	+	.....	.....	.....	.....	.....	.....	.....	10	.....	.....	.....	.....	17	.....
	C57BL/10–*H-2^{d}*	7	*48*	*64*	16	.....	36	39	.....	.....	.....	0	.....	.....	30	20	36
	C57BL/6–*H-2^{d}*	.....	.....	+	.....	.....	.....	.....	.....	.....	28	.....	.....	.....	7	.....	.....
H-2$^{d'}$	YBL/Rr	10	39	36	19	.....	36	.....	.....	.....	.....	17	.....	.....	7	33	33
H-2^{k}	C57BR/a	6	.....	11	.....	+	+	.....	.....	–	7	.....	.....	47	11	23	.....
	C57BR/cd	0	.....	12	.....	.....	.....	*54*	.....	.....	.....	12	13	–	11	18	.....
	CBA	0	.....	.....	*47*	.....	.....	.....	.....	61	.....	18	.....	.....	7	8	.....
	C3H	.....	.....	.....	.....	.....	+	.....	.....	.....	.....	.....	.....	.....	.....	.....	–
	ST	.....	11	17	+	*53*	.....	*52*	.....	.....	33	.....	.....	.....	6	3	.....
H-2$^{k'}$	AKR	15	23	10	29	.....	.....	52	.....	.....	12	.....	.....	.....	35	–	.....
H-2^{b}	C57BL/6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	+	.....	*60*	24	29	.....
	C57BL/10	7	7	.....	.....	.....	.....	.....	.....	–	.....	.....	+	.....	.....	.....	.....
	C57L	22	.....	0	.....	26	.....	.....	.....	.....	+	.....	.....	*67*	12	.....	.....
	LP	11	.....	.....	27	.....	.....	.....	.....	.....	*59*	*58*	*68*	.....	11	.....	.....
	129/Rr	14	–	.....	3	.....	.....	.....	.....	.....	.....	*61*	–	.....	11	28	.....
	A–*H-2^{b}T*	.....	.....	.....	.....	.....	.....	.....	.....	.....	*50*	.....	*47*	.....	.....	.....	.....
H-2^{p}	P	11	10	.....	.....	.....	.....	.....	.....	.....	0	.....	.....	.....	.....	.....	.....
H-2^{q}	DBA/1	.....	.....	18	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	4	.....	.....
H-2^{r}	RIII/Wy	18	.....	13	29	.....	.....	37	.....	.....	.....	8	.....	.....	8	8	.....

*Table also contains data from two additional publications (3, 7) not included in table 2. Where more than 95 percent of the mice died, + is entered instead of the recombination percent; where fewer than 21 percent of the mice died, – is entered instead of the recombination percent. Values in italics are those obtained where two stocks in the same allelic group were tested against each other.

H-2^{dk}. The first cross (case 1, table 2) tests strain DBA/2 against strain A. Since there is linkage, we conclude that strain DBA/2 is not *H-2*^{dk}, and assign to its *H-2* allele the symbol *H-2*^{d}. Strain DBA/2 may therefore be regarded as the *type strain* for allele *H-2*^{d}. Stocks whose tests against each other show no linkage, and which are therefore presumed to carry the same *H-2* allele, are grouped together. Thus cases 1, 2, and 3 test strains DBA/2 and BALB/c, which both belong to the *H-2*^{d} allelic group. As the table progresses, any strain to be established as carrying a new allele must be tested against one or more strains from each allelic group previously established and must show linkage in these tests. Thus 10 crosses (cases 93 to 102 inclusive) were used to prove that strain RIII/Wy does not have any one of seven previously identified alleles, and can therefore be classified as having a new allele, *H-2*^{r}.

In order to insure consistency in the few borderline cases, a set of rules was drawn up to govern the conclusion drawn from each type of result. These rules are given in appendix 1.

In discussing the evidence, it will be convenient to divide the crosses into three groups: A. Those showing no evidence of linkage; B. those showing clear evidence of linkage; C. those in which the result is ambiguous or uncertain.

A. *Crosses Showing No Evidence of Linkage.*—These are the crosses taken as evidence that the two strains under test carry the *same H-2* allele. Twenty-five crosses fall in this category. In some cases (*e.g.* case 3, DBA/2 tested against BALB/c) all mice succumbed to the tumor. These cases are valid evidence that the two strains being tested carry the same *H-2* allele only if it is assumed that a tumor bearing one allele will not grow, or will grow only infrequently, in a strain carrying another. That this assumption is justified, is indicated by the consistency of various check tests to be described below.

In a more numerous group of cases there were some survivors, but more than 46 percent (and usually more than 50 percent) crossing over. An example is case 26, C3H tested against ST, which gave a ratio of 16:7:15:4, or 73.8 percent mortality and 52.4 percent crossing over. A few cases (*e.g.* case 19, C3H tested against C57BR/cd, ratio 12:40:6:27) gave considerably less than 50 percent mortality.

The validity of the interpretation that absence of linkage establishes the occurrence of the same allele in two strains is indicated by the consistency of the results. There are four types of checks:

1) Two strains can be tested against each other in two ways, first with one as tumor donor, then with the other as tumor donor. The results are always consistent. Thus C57BL/6 tested against LP with C57BL/6 as the donor gave 11:5:11:0, and with LP as the tumor donor, 7:14:4:5. In both cases there is more than 50 percent recombination.

2) Where there are more than two strains in an allelic group, they can be tested against each other in a variety of combinations. Again the results, with rare exceptions to be pointed out later, are always consistent. Thus the 6 strains in the *H-2*^{b} group were tested in the following combina-

tions, and always gave either 100 percent mortality (3 cases) or more than 46 percent crossing over (10 cases): C57BL/10 against C57BL/6, C57BL/6 against C57L, C57L against C57BL/10, C57BL/6 against LP, LP against C57BL/6, C57BL/10 against LP, C57L against LP, LP against C57L, C57BL/10 against 129/Rr, C57L against 129/Rr, C57BL/6 against A–*H-2*^{b}*T* (made with two different stocks in the third position), C57L against A–*H-2*^{b}*T* (tumor donor named first in every case). Other examples of intragroup consistency in tests involving *H-2*^{d} have been reported in other papers (*3*, *7*).

3) With three exceptions (cases 32, 33, and 53) discussed further below, no strains in one allelic group have ever failed to show linkage with all strains in any other allelic group, wherever tests have been made. Thus in the case of the *H-2*^{d} and the *H-2*^{k} groups the following cross tests were made and showed linkage in every case: BALB/c against C57BR/a, BALB/c against C57BR/cd, CBA against DBA/2, DBA/2 against ST, BALB/c against ST.

The consistency with which linkage occurs in tests between allelic groups, and fails to occur in tests within an allelic group, is easily visualized from the condensed summary in table 3. Note how the results of intra-allelic tests (figures in italics) all show a recombination percent of 47 or greater. Note also that cases with mortality exceeding 95 percent (indicated by plus signs), all fall in these same groups.

4) The component test, discussed below, has given completely consistent results in determinations based on the complementary nature of groups *H-2*^{d} and *H-2*^{k}.

B. *Crosses Showing Clear Evidence of Linkage.*—There are 65 cases in this category, with recombination percents ranging from 0 to 37.7. A test of significance shows a *P* value of less than 0.01 for 61 of these; for the remaining 4 the value falls between 0.01 and 0.05. For the former group we may conclude that the occurrence of *different H-2* alleles in the two stocks under test is proved beyond reasonable question. The conclusion is the same, but a little less firmly established, for the latter four.

Since the incidence of crossing over between *T* and *H-2* is in the order of 5 to 10 percent, the occurrence of recombination values up to 37.7 percent requires explanation. This point is discussed under C.

C. *Crosses Giving Ambiguous or Uncertain Results.*—These include crosses in which the percentage of mice dying from the tumors was less than 21 (cases 31, 43, 54, 61, 64, 89, 93, 98), crosses in which the recombination percent suggests linkage but in which the departure from 50 percent recombination is not significant (cases 6, 15, 34, 75, 99), and crosses which lead to a conclusion in conflict with the conclusion indicated by some other cross or crosses (cases 32, 33, 53). It will be convenient to discuss these in terms of the different factors that can lead to distortion of or vagueness in the results.

1) Normal overlapping of Fu *and* T.—It is well established that mice genetically *Fu* not infrequently have normal tails (*11*). Such animals are called normal overlaps. The frequency of this phenomenon is much higher

in crosses involving certain strains than it is in others (Mrs. Sally Lyman Allen, unpublished data). It also occurs, though less commonly, with *T*, the genetic background again being an important factor. Strain AKR genes are particularly favorable to the expression of the two tail characters, strain C57BR/a and probably strain YBL genes particularly unfavorable (Allen and Snell, unpublished data). In cases 1, 4, 8, 27, 36, and 51 (table 2), some survivors with normal or questionable tails were mated to strain AKR as a means of determining their actual genotype. Of 7 such animals from crosses involving *Fu*, 3 were found to be genetically normal, 4 genetically *Fu*. In the one cross involving *T* (case 4, A tested against YBL), 3 out of 3 animals were proved to be *T*. Undoubtedly many more such cases would have been found had more animals been tested.

Since the normal overlaps usually fall in a crossover class, the net effect of such cases is to increase the apparent recombination percent.

2) *Differential mortality.*—It is well known that in a segregating generation, mortality is frequently higher among individuals of the mutant genotypes than among their normal sibs. Among the mutant genes used in our crosses, *Ki* is probably particularly subject to this effect. The ratio of 14 *Ki* to 28 normal-tailed mice obtained in cross 34 may be a manifestation of this differential mortality.

3) *The occurrence of false positives.*—It is well known that certain transplantable tumors, particularly old and virulent [5] ones which have been carried through many transplant generations, will occasionally grow in foreign-strain and hence presumably resistant animals. Numerous examples of this are given in the accompanying paper by Snell, Russell, Fekete, and Smith (*12*). There is good reason to suppose that a similar phenomenon has occurred in many of the linkage crosses listed in table 2. Thus case 47, C57BR/cd tested against C57L with a resulting ratio of 32:5:13:19, shows 45 positives to 24 negatives. This is greatly in excess of the expected 50 percent. Such cases have led us to coin the term *false positives*, defined as animals which are genetically resistant but which nevertheless succumb to progressive tumor growth. As we shall point out below, the incidence of false positives is probably, in part at least, a function of the tumor employed.

False negatives may also occur. MacDowell and Richter (*13*) raised 50 backcross mice from a cross that typically gave an approximation to a one-factor ratio and tested them genetically for susceptibility and resistance before inoculating the backcross mice themselves. Twenty-five mice—exactly half the group—bred like susceptibles, but only 17 of these succumbed to the tumor. The remaining 8 fell in the class that we would call false negatives.

To what extent the occurrence of false negatives is determined by environmental factors and to what extent by genetic factors is still an

[5] "Virulence" as applied to transplantable tumors is a term implying differences of degree. Several measures are possible, *e.g.* rate of growth in the native strain, capacity to grow in foreign strains, number of "factors" required for growth in hybrid generations (*14*), tendency to metastasize. There is probably some correlation between these different measures. As used in this and the two accompanying papers, the term connotes principally the first three qualities.

unanswered question, but modifying genes or minor histocompatibility genes almost certainly play a part. Probably no sharp line can be drawn between crosses giving false negatives and crosses giving two-factor ratios (see section 4 below).

The occurrence of false positives and false negatives, like the occurrence of normal overlaps, tends to raise the recombination percent to a value higher than that expected from genetic crossing over alone.

4) The segregation of histocompatibility genes other than H-2.—The *H-2* locus is only one of many histocompatibility loci (*14*, *15*). Most crosses of the type used in the present tests probably segregate for 4 or 5 or more such loci. How many of these express themselves in a given cross is a function, among other things, of the tumor inoculated. The typical ratio, where one gene is acting, is 50 percent deaths to 50 percent recoveries; for two genes, 25:75; for three genes, 12.5:87.5.

An analysis of the frequency with which different percentages of mortality occurred among the 101 crosses of table 2 is presented in table 4. The first two rows of data show the results, respectively, for crosses that produced fewer than 35 mice, and for crosses that produced 35 or more mice. In general, the former were crosses that gave clear results and were therefore terminated after 25 to 35 animals had been raised; the latter were crosses whose significance could only be determined with larger numbers.

It will be seen that the first group has a clear mode in the 45.9 to 54.1 percent mortality range. The ratio for many of these crosses is a good approximation to the 50:50 ratio expected in a one-factor cross. There are also a few crosses which gave so few deaths from tumor inoculation as to be valueless and which were dropped early on that account, and 6 crosses (all intra-allelic tests) giving 95.7 to 100 percent mortality.

The second group, "35 or more mice per test," has a mode at 54.2 to 62.4 percent mortality, and a second one at 12.7 to 29.2. The former group probably represents single-factor crosses distorted by the occurrence of "false positives," and the latter probably represents crosses segregating for 2 or more histocompatibility loci.

Line 3, "crosses not segregating at the *H-2* locus," is notable for the absence of any mode near the 50 percent level. Evidently in no case in these crosses was a single gene segregating whose presence or absence was the determining factor in susceptibility or resistance. There is, however, a possible mode at 21.0 to 29.2 percent, indicating that in certain cases susceptibility and resistance may have been determined by the joint action of two factors.

The fourth line, showing crosses which *were* segregating at the *H-2* locus, serves to emphasize the mode at the 50 percent level in these cases, but again there is a lower mode at 12.7 to 20.9 percent.

The significance of the different ratios obtained is discussed further in the next section. Suffice it to point out here that good two-factor ratios were notable by their absence. The expected ratio with two histocompatibility genes segregating, one of which is linked with short tail

TABLE 4.—*The frequency with which different percentages of mortality occurred among the 101 linkage tests*

Groups of crosses	Percentage mortality from progressive tumor growth													Totals
	0 to 4.3	4.4 to 12.6	12.7 to 20.9	21.0 to 29.2	29.3 to 37.5	37.6 to 45.8	45.9 to 54.1	54.2 to 62.4	62.5 to 70.7	70.8 to 79.0	79.1 to 87.3	87.4 to 95.6	95.7 to 100	
Crosses that produced fewer than 35 mice per test	1	1	1	0	9	5	15	7	2	2	1	0	6	50
Crosses that produced 35 or more mice per test	0	1	4	4	0	7	7	10	8	7	0	1	2	51
Crosses not segregating at the *H-2* locus	0	0	1	3	2	1	0	1	0	4	1	1	8	22
Crosses segregating at the *H-2* locus	1	2	4	1	7	11	22	16	10	5	0	0	0	79

with 5 percent crossing over, is 19:21:1:39. In an accompanying paper (7), Snell and Borges report approximations to this ratio in tests similar to those reported here, but none are to be found in the present group of tests.

While there were no clear two-factor ratios, some tumors did give significantly less than 50 percent mortality in some crosses, indicating that more than one histocompatibility gene was acting. Possible differences between the tumors in this respect can be seen by reference to the last column of table 1, which gives the "fraction of crosses giving percent + >37.5." It will be seen that tumors 15091a, S653, S636, S629, and S620 gave more than 37.5 percent deaths in all crosses. These are consistently one-factor tumors. At the other extreme, tumor D22 gave more than 37.5 percent deaths in only 1 of 4 crosses. This means that in at least 3 crosses more than one histocompatibility gene was acting to influence the growth of this tumor. However, as the next section will help to emphasize, we do not mean to imply that there was necessarily a sharp correspondence between the presence or absence of histocompatibility genes other than *H-2* and the growth or failure to grow of the tumor.

5) *The role of the tumor in disturbed ratios.*—If *a*) a cross is segregating for *H-2*, if *b*) no other histocompatibility gene is acting, and if *c*) the crossover value between *H-2* and *T* is 5 percent, then the expected ratio is:

normal tail		*short tail*	
+	−	+	−
19	1	1	19

or with 10 percent crossing over:

9	1	1	9

It became apparent early in the linkage tests that certain tumors gave ratios closer to the above than did others, and that this difference between tumors was not merely a matter of certain tumors behaving as two-factor or three-factor tumors.

To get a numerical measure of this difference between tumors, we have calculated for each tumor a combined crossover percent for all crosses where linkage should be clearly manifest. The proviso that linkage should be clearly manifest rules out *a*) crosses not segregating at the *H-2* locus, *b*) crosses not segregating for major *H-2* differences (see below), and *c*) crosses in which fewer than 37.5 percent of the mice were positive and in which, therefore, some one or more histocompatibility loci in addition to *H-2* were presumably active. The results are given in table 1, column 6. The calculated crossover percents range from 12.1 for strain P tumor S637, to 47.3 for strain LP tumor D22. The former value is a good approximation to the theoretical crossover percent. Any excess in the recombination class can probably be entirely or almost entirely accounted for by the occurrence of normal overlaps among genetically *Fu* (or *T*) mice. The latter value, 47.3 for tumor D22, obviously constitutes a major discrepancy.

A search for factors to account for the aberrant results given by a few of the tumors reveals a correlation between such results and the tendency of tumors to give or not to give regressions.

Since the size of all tumor transplants was scored weekly, beginning one to three weeks after inoculation, we have available data on regressions which can be treated in two ways, as follows.

(*a*) The tumors can be classified according to the maximum size attained by the transplants prior to regression. Column 7 of table 1 gives, in terms of our arbitrary grading system, the largest size recorded for each tumor among mice where regression rather than progressive growth was the ultimate fate of the transplant. The values range from 7.5 for tumor S637 to 0 for tumor S620. A tumor of grade 7.5 is a very large tumor, probably something in the order of 10 cc. in volume.

(*b*) Each mouse that survived can be classed as either negative at all checks, or as showing a tumor that regressed. We can then calculate for each tumor the percent of regressions that occurred among surviving mice. This value is given in table 1, column 8, for each of the 16 tumors. Undoubtedly more regressions would have been detected and the values in column 7 correspondingly increased had all mice been observed as early as the first week after tumor inoculation, but there is no reason to suppose that the data as they stand are biased in favor of any one tumor. The values range from 73 percent regressions for tumor S621 to 0 percent regressions for tumor S620.

The records of tumor size were made primarily as a check on whether each death among inoculated animals was due to progressive tumor growth, or to disease or some accidental cause. Some indication as to whether the data are sufficiently accurate to use as a measure of certain inherent properties of the tumor can be obtained by calculating the correlation between the two sets of values defined above and given in table 1, columns 7 and 8 respectively. Such calculation shows that these two measures of the tendency of each tumor to give regressions are highly correlated, with $r=.83$. This is evidence that these two values measure some real property of the tumors.

These two values (table 1, columns 7 and 8) in turn are negatively correlated with the recombination percent (column 6) for each tumor. For the maximum size, $r=-.64$. This value is probably partly spurious, since the "good" tumors were used in more crosses than the "poor" ones, thereby increasing the chance of finding the infrequent cases in which unusually large tumors regressed. A correlation not subject to this objection was obtained by using for maximum size for each tumor an average of the maximum grade recorded for each cross (data not given). The correlation calculated with these values is $r=-.37$. For percent regressions among negative mice, $r=-.59$.

A "median survival time" was also calculated for each tumor (table 1, column 9). This was obtained by averaging for each cross the number of days between inoculation and death for mice that died from the tumor, and then, for each tumor, selecting the median value for all crosses in which the tumor was used. This value shows a correlation of 0.53 with the recombination percent.

These correlations provide evidence for the following generalizations.

Certain tumors, when used to inoculate mice of a segregating generation, give ratios indicative of almost complete concomitance between histocompatibility phenotype, as measured by positive or negative outcome of the graft, and histocompatibility genotype. These are "good" tumors for genetic studies. Among the tumors we used, S637, C954, P1534, 15091a, S653, S621, and C1498 fall in this category. 6C3HED is probably also a "good" tumor, but gave only about 21 percent positives in the two crosses in which it was used where *H-2* was segregating, indicating the concurrent action of other genes.

Other tumors fail to produce a close concomitance between histocompatibility phenotype and genotype. Or stated in another way, they give numerous "false positives" and "false negatives." Tumors D1905, S629, S674, and especially S620 and D22, fall in this category. Intermediate in its behavior is tumor dbrB. Tumor S636 (C57BR/a origin) gave a recombination percent of 26.8, but is possibly a "better" tumor than this value would indicate since much of the disturbance here may be due to the tendency toward normal overlapping of *T* and *Fu* on a C57BR/a background (note, for example, the deficiency of short-tailed mice in cases 10, 34, and 97). Except for case 97, however, this tumor consistently killed more than 50 percent of the mice.

Tumors that tend to produce regressions are, in general, desirable tumors for genetic studies. With these tumors, histocompatibility phenotype and histocompatibility genotype show a sharp correspondence. Notable in this respect are strain P tumor S637 and strain BALB/c tumor S621, both of which gave not only many regressions but also frequent instances of rather large growths that regressed.

At the other extreme is CBA tumor S620, which gave no regressions whatsoever. With this tumor, once growth started it proceeded slowly but inevitably to the death of the host. This was almost if not quite the "worst" tumor genetically. The results obtained with LP tumor D22 are discussed in section III.

The relation between the occurrence of regressions and the genetic behavior of a tumor indicated by columns 6, 7, and 8 of table 1 is perhaps not surprising. The regression of large growths may be taken to indicate a sensitivity to those mechanisms within the host which resist foreign tissue grafts.

It is interesting to inquire whether there is any correlation between the histologic type of a tumor and its behavior in genetic tests. The two leukemias and the one histologically related lymphosarcoma used in these tests are all "good" tumors. It is noteworthy that with one of these leukemias, C1498, Borges and Kvedar (*5*), in extensive tests, obtained genetic ratios very close to the theoretical expectations. Aside from this possible significance of the leukemias and the lymphosarcoma, our data do not give any clues as to the significance of histologic type.

The significance of the correlation between recombination percent and median survival time is not entirely clear. In part it is due to the fact that the leukemias that stand high on the list as "good" tumors are also

the most rapidly growing tumors. We have pointed out previously (*3, 8*) that genetically resistant animals that succumb to the tumor (false positives) sometimes survive longer than genetically susceptible animals. Also interesting in this connection is the observation of Klein (*16*) that rapidly growing tumors with a low median survival time are more apt to prove convertible to the ascites form than slowly growing tumors with a high median survival time. Can this mean that ascites tumors should be good genetic tumors?

6) The effect of weak H–2 *differences.*—Cases 5, 6, and 30 (table 2) constitute a special class. Case 5, YBL/Rr tested against BALB/c (mice inoculated with BALB/c tumor S621), gave 36.2 ± 6.6 percent recombination, 69.0 percent positives, and 56.9 percent short tail. Case 6, YBL/Rr tested against DBA/2 (mice inoculated with DBA/2 tumor P1534), gave 38.9 ± 5.9 recombination, 70.8 percent positives, and 37.5 percent short tail. Case 30, AKR tested against C57BR/a (mice inoculated with C57BR/a tumor S636), gave 28.7 ± 5.0 percent recombination, 70.3 percent positives, and 44.5 percent short tail.

In all these three cases the recombination percent is less than 50. It is significantly less for case 30, and for the combined results of cases 5 plus 6. The combination is legitimate here since, though these crosses involve different tumor-donating stocks, they both test YBL/Rr against allele *H-2*^{d}.

The recombination value for each of the three crosses, while less than 50 percent, is significantly greater than 10 to 12 percent, the maximum expected as the result of crossing over alone. This situation requires explanation.

Pertinent here also are cases 32 and 33 which, like case 30, test strain AKR against strains belonging to the *H-2*^{k} group. Whereas case 30, since it shows linkage, puts strain AKR *out* of the *H-2*^{k} group, cases 32 and 33 show no linkage and therefore put strain AKR *in* the *H-2*^{k} group. There is an apparent conflict in the evidence.

The component test, described in a later section of this paper, puts strain AKR in the *H-2*^{k} group, thereby again conflicting with the evidence from case 30.

The anomalous recombination values found in cases 5, 6, and 30 are evidently not due to histocompatibility genes besides *H-2* acting to give two-factor ratios. The percent mortality expected with two factors is 25, whereas in all these crosses the percent actually ran closer to 70. The values are not due to normal overlaps at the *T* locus. There is a probably significant deficit of short-tail mice in cross 6, but hardly enough in itself to cause the observed distortion of the ratio. The proportion of *T* mice in the other two crosses is a good approximation to the expected 50 percent. The anomalous values for cases 5 and 6, at least, are not due to the poor discrimination of histocompatibility genotype that appears when a "poor" tumor is used. S621 and P1534 are both "good" tumors. The status of S636, used in cross 30 (strain AKR tested against C57BR/a, allele *H-2*^{k}), is somewhat uncertain. However, we are here

faced with the fact that cases 32 and 33, which test AKR against CBA and C3H (both allele *H-2*^{k}), show no linkage, so the surprising thing is not that case 30 fails to show a close linkage, but that it shows any linkage at all.

Having ruled out two-factor ratios, normal overlaps of *T*, and the action of "poor" tumors as explanations of the high recombination values found in cases 5, 6, and 30, two possible explanations would appear to remain.

The first is that these crosses are not segregating for *H-2* but for some other locus, also on chromosome 9 but approximately 30 crossover units from *T* instead of 5 to 10 units. This explanation cannot be entirely ruled out at the present time, but it does not fit the available facts as well as a second alternative. For one thing, if such a locus existed, anomalous linkage ratios would be likely to appear much more frequently than they do.

The second alternative is that the *H-2* allele carried by strain YBL/Rr differs from *H-2*^{d} (the allele in BALB/c and DBA/2), and that the allele in strain AKR differs from *H-2*^{k} (the allele in C57BR/a), but that the differences are minor ones, appropriately indicated by assigning the symbols *H-2*$^{d'}$ and *H-2*$^{k'}$ respectively. The tumor graft thus does not differentiate consistently between mice of the genotypes *H-2*^{d} and *H-2*$^{d'}$ or *H-2*^{k} and H–2$^{k'}$, with a resulting distortion of the recombination fraction. In certain crosses and with certain tumors (*e.g.* cases 32 and 33), the distinction may be lost altogether.

As we shall point out below, alternative two receives considerable support from the component tests.

II. The Component Test

The component test consists of crossing two strains and inoculating the F_1 hybrid with strain A tumor 15091a. Growth is expected to occur if, and only if, one of the parent strains is *H-2*^{d}, the other *H-2*^{k} (*4*).

The results of inoculating F_1 hybrids from 28 crosses are shown in table 5. The first 9 crosses, involving 8 different inbred strains, are of the type *H-2*^{d} × *H-2*^{k}. It should be emphasized here that the initial classification of all strains is based on the linkage test. Of 189 tested F_1 hybrids, 188 succumbed to the tumor and 1 was negative. There is essentially complete agreement between the results of the linkage test and the component test.

The next cross is between strains YBL/Rr (*H-2*$^{d'}$) and C57BR/cd (*H-2*^{k}). Of 20 F_1 hybrids, 18 succumbed and 2 survived. It would seem legitimate to interpret this result as confirming the conclusion drawn from the linkage test that the allele in strain YBL/Rr is similar to but not identical with *H-2*^{d}.

The next 18 crosses involve 16 inbred strains and were made in a variety of combinations, excluding only the one combination *H-2*^{d} × *H-2*^{k}. Of 302 F_1 hybrids, 51 succumbed to the tumor and 251 survived, the percent susceptible ranging from 0 for five of the crosses to 54 for the cross C57BR/a × DBA/1. Three of the 5 highest incidences of death

TABLE 5.—*Data showing the susceptibility of mice of the genotype* H-2^d/H-2^k *to strain A tumor 15091a* (H-2^{dk}/H-2^{dk}), *and the resistance of mice of other genotypes. Susceptibility to tumor growth indicated by* + (*mouse died of tumor*), *resistance by* −.

Cross	Genotype	No. of mice		Cross	Genotype	No. of mice	
		+	−			+	−
BALB/c × C57BR/a.	*H-2^d/H-2^k*	17	0	BALB/c × YBL/Rr.	*H-2^d/H-2$^{d'}$*	0	13
" × C57BR/cd	"	15	1	" × P.....	*H-2^d/H-2^p*	1	11
" × CBA.....	"	25	0	" × DBA/1...	*H-2^d/H-2^q*	2	15
" × C3H.....	"	20	0	" × RIII/Wy.	*H-2^d/H-2^r*	0	32
" × ST.....	"	30	0	DBA/2 × DBA/1...	*H-2^d/H-2^q*	1	10
DBA/2 × CBA.....	"	9	0	AKR × C57BL/6..	*H-2$^{k'}$/H-2^b*	0	20
" × ST.....	"	16	0	CBA × ".....	*H-2^k/H-2^b*	2	9
BALB/c × AKR....	*H-2^d/H-2$^{k'}$*	25	0	" × C57L.....	"	3	7
DBA/2 × AKR.....	"	31	0	" × C57BR/a..	*H-2^k/H-2^k*	2	8
YBL/Rr × C57BR/cd.	*H-2$^{d'}$/H-2^k*	18	2	" × P.....	*H-2^k/H-2^p*	2	11
				" × DBA/1....	*H-2^k/H-2^q*	5	23
				C3H × ST.....	*H-2^k/H-2^k*	3	16
BALB/c × C57BL/6.	*H-2^d/H-2^b*	1	22	" × RIII/Wy..	*H-2^k/H-2^r*	0	12
" × LP.....	"	0	3	C57BR/a × DBA/1.	*H-2^k/H-2^q*	14	12
" × 129/Rr...	"	6	17	C57BR/cd × "...	"	9	10

occurred in crosses involving DBA/1 as one parent and an *H-2^k* line as the other. Possibly this means that the allele in strain DBA/1 is akin to *H-2^d*. On the other hand it has been pointed out that this strain shows a unique susceptibility to tumor transplants in general (*12*).

The 51 positive animals from these 18 crosses must be classed as "false positives." They do not constitute a major exception to the rule that F_1 hybrids not *H-2^d/H-2^k* will not grow strain A tumor 15091a. Again the component test completely confirms the linkage test.

III. Inconsistencies in the Data

The one major inconsistency is case 53, strain LP tested against strain C57BR/a, with LP the tumor donor. No linkage was evident here (recombination percent 47.4±6.6), pointing to the conclusion that LP and C57BR/a carry the same *H-2* allele. However, the reciprocal cross (LP tested against C57BR/a, C57BR/a the tumor donor, case 52, table 2) gave a recombination value of 27.4±6.4. For reasons already pointed out, a significant linkage carries more weight than absence of linkage. We may therefore conclude that strain LP does not carry allele *H-2^k*. This is confirmed by cases 55, 56, 57, 58, and 59, which all agree in putting strain LP in group *H-2^b*.

The inconsistency in this case can be attributed to the fact that LP tumor D22 is a "poor" tumor, and also in part perhaps to a large number of *T* overlaps (tail ratio, 37 normal tail to 20 short tail) occasioned by the presence of C57BR/a genes.

Other inconsistencies involve the *H-2^k* and *H-2$^{k'}$* strains. Case 30 (AKR tested against C57BR/a, 28.7±5.0 percent recombination) proves that strain AKR does not have the allele *H-2^k* carried by strain

C57BR/a, and hence can be assigned the allelic symbol *H-2$^{k'}$*. However, cases 32 and 33, AKR tested against CBA and C3H respectively, gave results which *would* put AKR in the *H-2^{k}* group.

It is clear from case 30 that strains C57BR/a and AKR do not carry identical alleles. Beyond this we cannot go at the present time. An examination of table 3 shows that it is essentially as consistent to put strain C57BR/a, and perhaps also the related strain C57BR/cd, in one group, and CBA, C3H, and perhaps ST along with AKR in the other, as to make the alternative grouping indicated in the table.

The original *H-2^{k}* allele was identified in line K8 (*4*), a noninbred line carrying kinky tail. A test of K8 against C57BR/a (case 34) gave 35.7 ± 7.7 percent recombination. This indicates that the allele in C57BR/a may not be identical with the original *H-2^{k}* allele. However, since line K8 has been dropped, it would seem advisable from now on to treat strain C57BR/a as the type strain for this allele.

In a few cases the type strain of a new allelic group was not tested against YBL/Rr (the type strain for allele *H-2$^{d'}$*) or AKR (the type strain for allele *H-2$^{k'}$*). Specifically, strains C57BL/6 and AKR were not tested against YBL/Rr, and RIII/Wy was not tested against AKR. Also DBA/1 gave ambiguous results with AKR due to the large number of negatives. However, strain C57BL/10 (closely related to C57BL/6) was tested against YBL/Rr and gave clear linkage. Also, both linkage tests and component tests were so clear in placing C57BL/6 outside the *H-2^{d}* group and AKR in the *H-2^{k}* group that it seems most unlikely that either is *H-2$^{d'}$*, and they so clearly placed RIII/Wy and DBA/1 outside the *H-2^{k}* group that it seems most unlikely that either is *H-2$^{k'}$*.

Discussion

An interesting point brought out by this study is the absence of any complete recessive among the alleles at the *H-2* locus. As pointed out elsewhere (*3*) this finding, while contrary to the classical theory of transplantation (*14*), is in accord with experience with blood groups.

There is no reason to suppose that the list of alleles so far identified at the *H-2* locus is complete. Additional alleles will almost certainly be found as more stocks are tested. It is quite possible also that additional components comparable to the *d* and *k* components will be found. It may, however, prove more practical to analyze these by serological methods than by the strictly genetic methods so far employed.

As knowledge of the locus increases, the nomenclature system employed at present may prove unsatisfactory. However, it would appear advisable to let any revision wait until there is reason to suppose that whatever new system is adopted will be final.

Summary and Conclusions

The histocompatibility-2 or *H-2* locus in the mouse is a locus determining susceptibility and resistance to tumor (and presumably also normal tissue) transplants. It lies on chromosome 9, closely linked with the

TABLE 6.—*Alleles at the histocompatibility-2 locus and their distribution in 22 inbred strains*

$H\text{-}2^{dk}$*	$H\text{-}2^{d}$	$H\text{-}2^{d'}$	$H\text{-}2^{k}$	$H\text{-}2^{k'}$	$H\text{-}2^{b}$	$H\text{-}2^{p}$	$H\text{-}2^{q}$	$H\text{-}2^{r}$
A	DBA/2 BALB/c C57BL/6–*H-2*^{d} C57BL/6Ks–*H-2*^{d} C57BL/10–*H-2*^{d}	YBL/Rr	C57BR/a C57BR/cd† CBA† C3H† ST†	AKR	C57BL/6 C57BL/10 C57L LP 129/Rr A–*H-2*^{b}*T*	P	DBA/1	RIII/Wy

*Note that allele $H\text{-}2^{dk}$ was previously called $H\text{-}2^{d}$.
†Whether these strains are $H\text{-}2^{k}$ or $H\text{-}2^{k'}$ is not established by the evidence so far available.

genes fused tail (*Fu*), kinky tail (*Ki*), and short tail (*T*). This linkage has been used to analyze the alternative forms or alleles that exist at this locus, and to map their distribution in twenty-two inbred strains.

The results to date are summarized in table 6. Nine alleles have been identified, $H\text{-}2^{dk}$, $H\text{-}2^{d}$, $H\text{-}2^{d'}$, $H\text{-}2^{k}$, $H\text{-}2^{k'}$, $H\text{-}2^{b}$, $H\text{-}2^{p}$, $H\text{-}2^{q}$, and $H\text{-}2^{r}$. Alleles $H\text{-}2^{d}$, $H\text{-}2^{k}$, and $H\text{-}2^{b}$ are apparently the most widespread, occurring in 4, 5, and 6 stocks respectively. The other alleles have so far been found in only 1 stock each.

Alleles $H\text{-}2^{d}$ and $H\text{-}2^{k}$ are of particular interest in that the hybrid $H\text{-}2^{d}/H\text{-}2^{k}$ is susceptible to strain A ($H\text{-}2^{dk}/H\text{-}2^{dk}$) tumor 15091a. This provides an independent check for the classification of alleles made by means of the linkage test.

Possible limitations of the methods employed are discussed. Evidence is presented that histocompatibility phenotype, as determined by growth or failure to grow of tumor transplants, does not always coincide with histocompatibility genotype. This is more often the case with certain transplantable tumors than with others. A correlation is found to exist between the exactness of ratios given by tumors when inoculated in mice from appropriate crosses, and their tendency to produce regressions. It is concluded that tumors with a marked tendency to give growth followed by regression are particularly suitable for genetic studies.

References

(*1*) DUNN, L. C., and CASPARI, E.: A case of neighboring loci with similar effects. Genetics 30: 543–568, 1945.

(*2*) SNELL, G. D.: Preliminary data on crossing over between *H-2* and *Fu*, *Ki*, and *T* in the mouse. Heredity 6: 247–254, 1952.

(*3*) SNELL, G. D., and HIGGINS, G. F.: Alleles at the histocompatibility-2 locus in the mouse as determined by tumor transplantation. Genetics 36: 306–310, 1951.

(*4*) SNELL, G. D.: A fifth allele at the histocompatibility-2 locus of the mouse as determined by tumor transplantation. J. Nat. Cancer Inst. 11: 1299–1305, 1951.

(*5*) BORGES, P. R. F., and KVEDAR, B. J.: A mutation producing resistance to several transplantable neoplasms in the C57 black strain of mice. Cancer Research 12: 19–24, 1952.

(6) BORGES, P. R. F., KVEDAR, B. J., and FOERSTER, G. E.: Development of an isogenic resistant subline of mice (C57BL/6–*H-2*^{d}) from the C57BL/6 strain. In preparation.

(7) SNELL, G. D., and BORGES, P. R. F.: Determination of the histocompatibility locus involved in the resistance of mice of strains C57BL/10-*x*, C57BL/6-*x*, and C57BL/6Ks to C57BL tumors. J. Nat. Cancer Inst. 14: 481–484, 1953.

(8) GORER, P. A., LYMAN, S., and SNELL, G. D.: Studies of the genetic and antigenic basis of tumour transplantation. Linkage between a histocompatibility gene and "fused" in mice. Proc. Roy. Soc., London, s. B. 135: 499–505, 1948.

(9) BITTNER, J. J.: The experimental determination of an invisible mutation. Paper Mich. Acad. Sci. 11: 349–351, 1929.

(10) DUNHAM, L. J., and STEWART, H. L.: A survey of transplantable and transmissible animal tumors. J. Nat. Cancer Inst. 13: 1299–1377, 1953.

(11) REED, S. C.: The inheritance and expression of fused, a new mutation in the house mouse. Genetics 22: 1–13, 1937.

(12) SNELL, G. D., RUSSELL, E., FEKETE, E., and SMITH, P.: Resistance of various inbred strains of mice to tumor homoiotransplants, and its relation to the *H-2* allele which each carries. J. Nat. Cancer Inst. 14: 485–491, 1953.

(13) MACDOWELL, E. C., and RICHTER, M. N.: Studies on mouse leukemia. V. A genetic analysis of susceptibility to inoculated leukemia of line I. Biol. Zentralblatt 52: 266–279, 1932.

(14) LITTLE, C. C.: The genetics of tumor transplantation. *In* The Biology of the Laboratory Mouse (G. D. Snell, ed.). Philadelphia, The Blakiston Co., 1941, pp. 279–309.

(15) SNELL, G. D.: Methods for the study of histocompatibility genes. J. Genetics 49: 87–108, 1948.

(16) KLEIN, G.: Conversion of solid into ascites tumours. Nature 171: 398–399, 1953.

APPENDIX 1.—*Rules used for drawing conclusions from linkage data (table 2)*

Case	If	Conclusion is
A.....	% + $<$21.....	?
B.....	21 $\leqq$ % + $<$95, and	
1.....	d/σ $\geqq$ 2.58.....	U is not *H-2*^{a}.
2.....	2.58 $>$ d/σ $\geqq$ 2.05.....	U is not *H-2*^{a}?
3.....	2.05 $>$ d/σ, recombination % $<$46.....	?
4.....	2.05 $>$ d/σ, recombination % $\geqq$46.....	U is *H-2*^{a}.
C.....	% + $>$95.....	U is *H-2*^{a}.

% + = the percent dying from the tumor transplant.
d/σ = (50%−observed recombination %)/standard error.
U = the "unknown" strain, or the strain under test.
H-2^{a} = the *H-2* allele in the "known" strain.

Determination of the Histocompatibility Locus Involved in the Resistance of Mice of Strains C57BL/10-*x*, C57BL/6-*x*, and C57BL/6Ks to C57BL Tumors [1,2]

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The histocompatibility-2 or *H-2* locus in the mouse is described in the preceding paper (*1*). Nine alleles have been identified. These, with the inbred strains in which they occur, are: $H\text{-}2^{dk}$ (strain A), $H\text{-}2^{d}$ (strains BALB/c and DBA/2), $H\text{-}2^{d'}$ (strain YBL/Rr), $H\text{-}2^{k}$ (strains C57BR/a, C57BR/cd, CBA, C3H, ST), $H\text{-}2^{k'}$ (strain AKR), $H\text{-}2^{b}$ (strains C57BL/6, C57BL/10, C57L, LP, 129/Rr), $H\text{-}2^{p}$ (strain P), $H\text{-}2^{q}$ (strain DBA/1) and $H\text{-}2^{r}$ (strain RIII/Wy).

This paper is concerned with the analysis of strains C57BL/10-*x* (*2*), C57BL/6-*x* (*3*), and C57BL/6Ks,[5] to determine what *H-2* allele they carry. These strains are branches of strains C57BL/10 and C57BL/6 respectively. They are characterized by resistance to tumors indigenous to and growing 100 percent in strains C57BL/10 and C57BL/6.

Methods

The methods are the same as those described in full in the preceding paper. They are based on two tests.

1) *The linkage test.*—This utilizes a cross of the type $(M \times T) \times N$, where M and N are any two inbred strains and T is a strain carrying the gene for brachyury, *T* (or for fused, *Fu*). The offspring of this double cross are inoculated with a tumor from strain M. The occurrence of linkage, as manifested by an excess of survivors among brachyury or short-tail mice, is taken as evidence that strains M and N carry *different H-2* alleles, the absence of linkage as evidence that they carry the *same H-2* allele.

2) *The component test.*—In this test the strain being analyzed is crossed to strains of the genotype $H\text{-}2^{d}/H\text{-}2^{d}$ and $H\text{-}2^{k}/H\text{-}2^{k}$, and the F_1 hybrid

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[4] The authors gratefully acknowledge the technical assistance of Mr. Frederick Gabrielson and Miss Priscilla Smith.

[5] "Derived from non-pedigreed inbred mice bred at the Jackson Laboratory and shipped to the Sloan-Kettering Institute in Oct. 1947. The present line was started from these mice in Feb. 1948. The strain is now almost completely resistant to two standard transplantable C57BL/6 tumors (E0771, C1498)."—*Mouse News Letter* #9, July 1953.

inoculated with strain A tumor 15091a. This tumor will grow progressively in all F_1 hybrids if, and only if, their genotype is *H-2*^{d}/*H-2*^{k}. Hence if the known parent is *H-2*^{k} and the tumor grows, the unknown must be *H-2*^{d}.

Results

The results are summarized in tables 1, 2, and 3.

In the linkage test, where the strain in the N position was C57BL/10-*x*, and the strain in the M position carried alleles *H-2*^{b}, *H-2*^{dk}, *H-2*^{k}, *H-2*^{p}, *H-2*^{q}, or *H-2*^{r}, it will be seen that an association between *T* (or *Fu*) and resistance was invariably manifest (table 1, cases 1 to 10). The recombination values range from 0 to 38.8 percent.

TABLE 1.—*Data showing linkage, or absence of linkage, between* T (*or* Fu) *and resistance, among segregants from crosses involving C57BL/10-*x *and 10 other inbred strains. Susceptibility to tumor growth is indicated by* + (*mouse died of tumor*), *resistance by* −. *The tumor used in each case was native to the first strain listed under each cross.*

Case No.	Allele tested	Cross	Number of mice				Recombination percent
			++	+−	*T*+	*T*−	
1	*H-2*^{b}	(C57BL/10 × A-*T*) × C57BL/10-*x*.	12	0	0	18	0.0
2	*H-2*^{dk}	(A × A-*H-2*^{b}*T*) × C57BL/10-*x*....	15	1	1	10	7.4 ± 9.6
3	*H-2*^{k}	(C57BR/a × A-*T*) × C57BL/10-*x*..	12	2	3	14	16.1 ± 9.0
4	"	(C3H × A-*T*) × C57BL/10-*x*.....	12	18	1	18	38.8 ± 7.1
5	"	(CBA × A-*T*) × C57BL/10-*x*.....	7	0	9	9	36.0 ± 10.0
6	*H-2*^{p}	(P × F^h) × C57BL/10-*x*.....	10	11	1	10	37.5 ± 8.8
7	"	(P × A-*T*) × C57BL/10-*x*.....	20	11	3	13	29.8 ± 7.3
8	"	Total of cases 6 and 7.....	30	22	4	23	32.5 ± 5.6
9	*H-2*^{q}	(DBA/1 × A-*T*) × C57BL/10-*x*...	15	1	9	25	20.0 ± 7.1
10	*H-2*^{r}	(RIII/Wy × A-*T*) × C57BL/10-*x*..	17	18	10	33	35.9 ± 5.7
11	*H-2*^{d}	(BALB/c × A-*T*) × C57BL/10-*x*..	10	3	15	0	64.3 ± 9.4
12	"	(DBA/2 × A-*T*) × C57BL/10-*x*...	26	14	18	8	48.5 ± 6.2

For cases 1, 2, 3, and 9, testing alleles *H-2*^{b}, *H-2*^{dk}, *H-2*^{k}, and *H-2*^{q}, respectively, the recombination value falls short of 50 percent by an amount in excess of three times the standard error. For cases 4 and 5, testing allele *H-2*^{k}, the departure from 50 percent is not significant, but this allele is adequately tested by case 3. For case 7, testing allele *H-2*^{p}, the difference from 50 percent is 2.8 times the standard error. However, it is legitimate in this instance to combine cases 6 and 7, since these differ only in the marker gene employed, and the combined value (case 8) gives unquestionable proof that strain C57BL/10-*x* is not *H-2*^{p}. For case 10, testing allele *H-2*^{r}, the recombination value is 35.9±5.7. The difference from 50 percent is 2.47 times the standard error. This is a slightly less conclusive test than for the other alleles, but can probably be regarded as adequate. We may therefore conclude that strain C57BL/10-*x* carries an *H-2* allele which is not *H-2*^{b}, *H-2*^{dk}, *H-2*^{k}, *H-2*^{p}, *H-2*^{q}, or *H-2*^{r}.

Case 1 is of particular interest in that it tested strain C57BL/10-*x* against strain C57BL/10, the line from which it arose. The results here

are particularly clear—there are no crossovers in 30 mice. This proves that strains C57BL/10-*x* and C57BL/10 differ at the *H-2* locus.

An entirely different type of result was obtained when the strain in the M position carried allele *H-2*^{d} (cases 11 and 12, table 1). For strain BALB/c, 25 of 28 mice were susceptible. The three negative animals were normal-tailed, and hence fall in the recombination category. There was no evidence of linkage. For strain DBA/2, there were 44 positive animals and 22 negatives, but again no evidence of linkage (crossover percent=48.5±6.2). There was a deficiency of brachyury (*T*) mice, but this is not an infrequent occurrence, due probably to either differential mortality or the occurrence of normal overlaps.

Tests with BALB/c and DBA/2, the two previously identified *H-2*^{d} lines, therefore agree in placing strain C57BL/10-*x* in the *H-2*^{d} group.

Three crosses in table 1 are of interest in that they gave approximations to the ratio expected when growth of the tumor is controlled by two histocompatibility genes, one of which is linked with *T*. Assuming 5 percent crossing over between *H-2* and *T* the expected ratio is:

++	+−	T+	T−
19	21	1	39

It will be seen that cases 4, 8, and 10, with observed ratios of 12:18:1:18, 30:22:4:23, and 17:18:10:33, respectively, approximate the ratio expected for two factors more or less closely.

Results with the linkage test are confirmed by the component test (table 2, cases 1, 2, and 3). All of 10 F_1 hybrids from the cross C57BL/10-*x* × C57BR/a and all of 13 F_1 hybrids from the cross C57BL/10-*x* × CBA grew strain A (*H-2*^{dk}/*H-2*^{dk}) tumor 15091a. All of 10 F_1 hybrids from the cross C57BL/10-*x* × BALB/c were resistant. If strain C57BL/10-*x* is *H-2*^{d}, then hybrids from the first two crosses are *H-2*^{d}/*H-2*^{k} and should be susceptible, and hybrids from the last cross are *H-2*^{d}/*H-2*^{d} and should be resistant.

The linkage test was not used in the analysis of strain C57BL/6Ks. However, the component test places it unequivocally in the *H-2*^{d} category. All of 12 F_1 hybrids from the cross C57BL/6Ks × C3H grew 15091a, and all of 20 F_1 hybrids from the cross C57BL/6Ks × BALB/c were resistant (table 2, cases 4 and 5).

TABLE 2.—*Growth of strain A tumor 15091a* (H-2^{dk}/H-2^{dk}) *in* F_1 *hybrids*

Case No.	Cross	Genotype of hybrid	Number of mice	
			+	−
1	C57BL/10-*x* × C57BR/a	*H-2*^{d}/*H-2*^{k}	10	0
2	" × CBA	"	13	0
3	" × BALB/c	*H-2*^{d}/*H-2*^{d}	0	10
4	C57BL/6Ks × C3H	*H-2*^{d}/*H-2*^{k}	12	0
5	" × BALB/c	*H-2*^{d}/*H-2*^{d}	0	20

Strain C57BL/6-*x* was tested by three linkage crosses (table 3). There was linkage in tests with strains C57BL/6 and P, but no linkage in a test with strain BALB/c. Despite the limited number of crosses used, this is in our experience fairly conclusive evidence that strain C57BL/6-*x* is *H-2*^{d}.

TABLE 3.—*Data showing linkage, or absence of linkage, between* T *and resistance, among segregants from crosses involving C57BL/6-*x *and 3 other inbred strains. Susceptibility to tumor growth is indicated by* + *(mouse died of tumor), resistance by* −. *The tumor used in each case was native to the first strain listed under each cross.*

Case No.	Allele tested	Cross	Number of mice				Recombination percent
			++	+−	T+	T−	
1	*H-2*^{b}	(C57BL/6 × A–*T*) × C57BL/6-*x*....	28	7	11	18	28. 1 ± 6. 2
2	*H-2*^{p}	(P × A–*T*) × C57BL/6-*x*.....	10	2	0	18	6. 7 ± 9. 1
3	*H-2*^{d}	(BALB/c × A–*T*) × C57BL/6-*x*....	12	0	19	0	61. 3 ± 9. 0

Summary and Conclusions

Borges *et al.* (*2*, *3*) have presented evidence indicating that strains C57BL/10-*x* and C57BL/6-*x* probably arose from their respective parent strains, C57BL/10 and C57BL/6, by mutation at a single histocompatibility locus. The evidence here presented shows that the mutation involved was a change from *H-2*^{b} to *H-2*^{d}. Strain C57BL/6Ks likewise differs from strain C57BL/6 in that it is *H-2*^{d} instead of *H-2*^{b}. Since strains C57BL/6-*x* and C57BL/6Ks are possibly related, the mutant allele they carry may have a common origin. According to the rules for nomenclature of inbred strains of mice (*4*), the mutant strains may be designated hereafter as C57BL/10–*H-2*^{d}, C57BL/6–*H-2*^{d}, and C57BL/6Ks–*H-2*^{d}.

References

(*1*) SNELL, G. D., SMITH, P., and GABRIELSON, F.: Analysis of the histocompatibility-2 locus in the mouse. J. Nat. Cancer Inst. 14: 457–480, 1953.

(*2*) BORGES, P. R. F., and KVEDAR, B. J.: A mutation producing resistance to several transplantable neoplasms in the C57 black strain of mice. Cancer Research 12: 19–24, 1952.

(*3*) BORGES, P. R. F., KVEDAR, B. J., and FOERSTER, E. G.: Development of an isogenic resistant subline of mice (C57BL/6–*H-2*^{d}) from the C57BL/6 strain. In preparation.

(*4*) CARTER, T. C., DUNN, L. C., FALCONER, D. S., GRÜNEBERG, H., HESTON, W. E., and SNELL, G. D.: Standardized nomenclature for inbred strains of mice. Cancer Research 12: 602–613, 1952.

Resistance of Various Inbred Strains of Mice to Tumor Homoiotransplants, and Its Relation to the *H-2* Allele Which Each Carries [1, 2]

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A homoiotransplant or homograft is defined as a transplant or graft between inbred strains. As a rule, tumor homoiotransplants do not grow, or grow temporarily and then regress (*1*). There are, however, occasional exceptions.

It is the purpose of this paper to examine the frequency of these exceptions for 13 transplantable mouse tumors and 15 inbred host strains, and to seek some clues as to their explanation.

Materials and Methods

The strains and substrains of mice used were: A/Jax, A/GoldinJax, A/HeJax, A/WaJax, BALB/Jax, BALB/cJax, BALB/cAnJax, BALB/cSn, CBA/Jax, CBA/JSn, C3H/Jax, C3H/ScJax, C57BL/6Jax, C57BL/6JSn, C57BL/6HeJax, C57BL/6pJax, C57BL/6–*H-2*^{d}, C57BL/10Jax, C57BL/10ScSn, C57BR/aJSn, C57BR/cdJax, C57L/Jax, C57L/HeJax, DBA/1Jax, DBA/1Hu, DBA/2Jax, DBA/2Vi, DBA/2Wy, LP/Jax, ST/Jax. The origin of most of these strains and substrains has been reviewed elsewhere (*2*, *3*) and further discussion here would be superfluous. Because certain of the substrains within each strain are closely related and showed no significant differences in their response to the grafts, all strains and substrains will hereafter be grouped into 15 lines as follows: A, BALB, BALB/c, CBA, C3H, C57BL/6, C57BL/6–*H-2*^{d}, C57BL/10, C57BR/a, C57BR/cd, C57L, DBA/1, DBA/2, LP, and ST.

Some of these strains, even as thus grouped, are more closely related than others (*2*, *3*). Thus all the C57 strains constitute a family with common ancestry. Substrains 6 and 10 of strain C57BL were separated only after prolonged inbreeding and are similar in nearly all respects.

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[4] The authors gratefully acknowledge the technical assistance of Mrs. Elizabeth Woodworth and Mrs. Caroline Higgins.

Other related pairs are BALB and BALB/c, CBA and C3H, DBA/1 and DBA/2.

The tumors used were—strain A: Anaplastic carcinoma 15091a (mammary gland origin), neuroblastoma C1300, sarcoma I; strain BALB/c: fibrosarcoma S621; strain C3H: lymphosarcoma 6C3HED, adenocarcinoma C3HBA; strain C57BL/6: myeloid leukemia C1498, mammary carcinoma E0771 (from C57BL subline C57BL/6$_a$DBA/1); strain C57BR/a: fibrosarcoma S636; strain DBA/1: mammary carcinoma dbrB; strain DBA/2: lymphatic leukemia P1534; strain unknown: sarcoma 37, sarcoma 180.

All mice were inoculated subcutaneously by trocar on the right side. Inoculations of C57BL/6–*H-2*^{d} mice with tumors P1534, C1498, E0771, and dbrB constitute an exception. These mice (for data concerning which we are indebted to Dr. P. R. F. Borges) received grafts subcutaneously by trocar on both sides simultaneously.

Most of the inoculations were performed in the Inbred Nucleus of the Jackson Memorial Laboratory. For some data we are indebted to Dr. N. Kaliss. A few data have been taken from control groups of experiments performed by one of the authors.

The ages of the mice at the time of inoculation ranged from one to seven months. A tabulation of different age groups for each strain of host revealed no consistent pattern of susceptibility in relation to age. Age is therefore disregarded in the following presentation.

All inoculations, except for some of the tests with C1498 tumor summarized in table 2, were performed after August 7, 1948.

Results

The results are presented in table 1. The data from each interstrain test are given in the form of a fraction, the numerator being the number of mice dying from progressive tumor growths, the denominator the total number of mice inoculated. For each tumor in its native strain the table shows, instead of a fraction, the value 100 percent. This value is in every case based on considerable numbers of mice, and in nearly every case where results from several sublines are lumped, the native tumor was tested and grew 100 percent in each subline of the group. Thus the four A sublines, A/Jax, A/GoldinJax, A/HeJax, and A/WaJax, all included in the table under strain A, were individually tested with strain A tumor 15091a and all grew it without exception.

The 15 inbred strains are arranged in 5 groups according to the *H-2* allele they carry (*4*). The 11 tumors of known origin are arranged according to the 7 different strains in which they originated, and the strains in turn grouped according to their *H-2* allele. Sarcoma 37 and sarcoma 180, whose origins are unknown, constitute an additional group. The *H-2* groupings are separated by heavy lines.

When thus arranged, certain consistencies appear in the data.

As expected, where two strains were related, a tumor native to one tended to grow in the other. Thus both C57BL/6 tumors grew 100

TABLE 1.—*Fraction* of mice from 15 inbred strains and substrains dying from homografts of 13 transplantable tumors*

Donor strain	Tumor	Host strain and histocompatibility-2 group															
		H-2^{dk}	*H-2*^{d}				*H-2*^{k}					*H-2*^{b}				*H-2*^{q}
		A	BALB	BALB/c	C57BL/6–*H-2*^{d}	DBA/2	CBA	C3H	C57BR/a	C57BR/cd	ST	C57BL/6	C57BL/10	C57L	LP	DBA/1
A	15091a	100%	0/10	1/21	.....	5/30	1/10	0/21	0/10	0/10	5/11	0/20	0/10	0/10	0/10	4/10
	C1300	100%	0/10	15/40	.....	.....	.....	0/19	11/33	0/10	9/11	0/10	0/10	.....	.....	16/20
	Sarcoma I	100%	0/9	0/20	.....	.....	.....	0/22	0/20	0/10	0/9	0/21	0/12	0/10	.....	0/10
BALB/c	S621	.....	10/10	100%	.....	10/10	0/9	0/9	0/40	0/5	.....	0/20	.....	.....	0/10	12/20
DBA/2	P1534	0/23	3/10	25/51	0/34	100%	.....	0/24	.....	0/20	0/9	0/20	1/9	0/20	.....	2/10
C3H	6C3HED	.....	0/10	.....	.....	.....	10/10	100%	0/18	0/10	2/19	0/10	0/20	.....	0/10	8/10
	C3HBA	.....	.....	.....	.....	0/12	.....	100%	0/17	.....	0/10	0/10	.....	.....	.....	3/10
C57BR/a	S636	0/19	.....	.....	.....	1/10	4/14	0/10	100%	14/15	0/10	0/9	0/15	.....	.....	1/10
C57BL/6	C1498	0/39	0/11	0/22	3/393	0/33	1/7	0/19	0/12	0/10	0/10	100%	20/20	48/49	1/29	1/10
	E0771	5/29	0/10	0/19	23/102	1/10	.....	5/38	0/10	2/10	1/19	100%	11/11	20/20	9/9	9/10
DBA/1	dbrB	0/30	0/10	1/19	0/31	0/21	0/10	2/20	0/10	0/10	0/10	3/29	1/10	0/20	0/10	100%
?	Sarcoma 37	.....	.....	20/20	8/8	.....	.....	.....	.....	.....	.....	9/20	.....	.....	.....	10/10
	Sarcoma 180	11/31	10/10	40/41	9/9	19/19	5/10	15/21	.....	8/30	17/27	16/30	6/11	4/10	.....	20/20

*The data for each interstrain test are given in the form of the fraction: $\frac{\text{Mice dying from progressive tumor growths}}{\text{Total number of mice inoculated}}$. For each tumor in its native strain the table shows, instead of a fraction, the value 100%.

percent in strain C57BL/10, and C3H tumor 6C3HED grew 100 percent in strain CBA.

This was true, however, only when the related strains carried the same *H-2* allele. The C57BL strains fall into 3 of the 5 *H-2* groups: C57BL/6–*H-2*^{d} is *H-2*^{d}; C57BR/a and C57BR/cd are *H-2*^{k}; C57BL/6, C57BL/10, and C57L are *H-2*^{b}. This was strikingly reflected in the cross tests. Thus C57BL/6 tumor C1498 grew in 48 of 49 C57L mice, but in only 3 of 393 C57BL/6–*H-2*^{d} and not at all in C57BR/a and C57BR/cd mice; C57BR/a tumor S636 grew in 14 of 15 C57BR/cd but not at all in C57BL/6 or C57BL/10 mice.

More significant, perhaps, is the result of cross tests between less closely related strains. Here again the role of the *H-2* locus is apparent.

Two components, *d* and *k*, are recognized at the *H-2* locus. Strain A has both of these (is *H-2*^{dk}), the groups of strains with alleles *H-2*^{d} or *H-2*^{k} each have one component but not the other, and the strains with allele *H-2*^{b} have neither. This relationship is reflected in the fact that strain A tumors 15091a and C1300 grew in a total of 21 of 111 mice of the *H-2*^{d} strains, in 26 of 135 mice of the *H-2*^{k} strains, but in none of 70 mice of the *H-2*^{b} strains. The presence of both *d* and *k* components in the tumor and the absence of both in the host apparently produce a degree of difference which the tumor cannot easily overcome. Strain DBA/1 (*H-2*^{q}) constitutes an exception on which we shall comment later.

The action of the *H-2* locus again shows up in that homografts tend to grow better when made between strains with the same *H-2* allele than they do when made between strains with different *H-2* alleles. This is true even when the strains involved have no close common ancestry. Thus the highest percentage of takes of grafts of BALB/c tumor S621 in any foreign strain without close common ancestry was in DBA/2 mice (all of 10), of DBA/2 tumor P1534 was in BALB/c mice (25 of 51), of C57BR/a tumor S636 was in CBA mice (4 of 14), and of C57BL/6 tumor E0771 was in LP mice (all of 9). The relationship is shown less clearly by C3H tumor 6C3HED, which grew in 2 of 19 ST mice but also in 8 of 10 DBA/1 mice, and by C57BL/6 tumor C1498 which grew in 1 of 29 LP mice but also in 1 of 7 CBA and 1 of 10 DBA/1 mice.

Particularly interesting is the behavior of the so-called "nonspecific" tumors, sarcoma 37 and sarcoma 180. Actually these show a striking specificity in favor of *H-2*^{d} hosts. Thus sarcoma 180 grew in 78 of 79 mice of strains BALB, BALB/c, C57BL/6–*H-2*^{d}, and DBA/2, but in only 102 of 190 mice of all other strains. The only strain outside the *H-2*^{d} group giving higher than 71 percent growth was DBA/1, which gave 100 percent (all of 20).

The data with sarcoma 37 are similar so far as they go, but less extensive.

Most critical are the results with strain C57BL/6 and its isogenic mate, C57BL/6–*H-2*^{d}, which differ only in the *H-2* allele that they carry. There were 9 deaths among 20 C57BL/6 mice, and 8 deaths among 8 C57BL/6–*H-2*^{d} mice inoculated at the same time with sarcoma 37. For sarcoma 180 the figures were 16 of 30 and 9 of 9, inoculations again being

simultaneous. In this test the C57BL/6–*H-2*^{d} mice were slightly older than the C57BL/6 mice; hence any age differential was in the direction of lesser susceptibility of the *H-2*^{d} line.

Sarcomas 37 and 180 thus show a definite affinity for the *H-2*^{d} lines. This is most easily explained by assuming that these tumors arose in mice of the genotype *H-2*^{d}/*H-2*^{d}.

Strain DBA/1 is of interest in that it seems to be unique in its susceptibility to homografts from donor strains not closely related genetically. Excluding tests with sarcomas 37 and 180, it gave 56 deaths among 120 mice. The nearest approach to this among the other strains is found in strain BALB/c, in which 42 of 192 homoiotransplants from unrelated donors grew progressively. However, most of the deaths among the BALB/c mice are due to tumor P1534. This is a combination in which tumor and host, though not closely related genetically, share the same *H-2* allele. Leaving these out, the figure for the BALB/c mice becomes 17 deaths among 141 mice.

All DBA/1 mice inoculated with sarcomas 37 and 180 gave progressive growth, and there was progressive growth in 1 or more mice with all 10 of the other foreign-strain tumors except sarcoma I.

It is possible that one or two of the other strains tested less thoroughly than DBA/1 would show a comparable sensitivity to homografts if more extensive tests were made. However, the figures so far as they go do not point in this direction. Thus the related strain DBA/2 gave 1 death in 10 mice with E0771, whereas strain DBA/1 gave 9 deaths in 10 mice with this tumor.

Additional information in regard to the growth of tumor homografts is provided by a series of experiments run between June 1945 and July 1949 in which C57BL leukemia C1498 was inoculated in C57L mice. Table 2 shows the results obtained with untreated controls in this series of experiments.

It will be seen that during the first year results were nearly all negative, progressive growth of the transplant occurring in only 1 mouse in 68. At the next test, 4 months later, an abrupt change was evident—4 of 8 mice died from the tumor. Over the next 9 months a series of experiments

TABLE 2.—*Progressive increase in the lethality of C57BL/6 transplantable leukemia C1498 tested in C57L mice*

Date of inoculations	Fraction dying	Percent dying
6/8/45	1/20	5
10/12/45 to 5/24/46 (6 separate tests)	0/48	0
11/29/46	4/8	50
1/10/47	5/15	33
3/24/47	5/20	25
7/7/47	6/10	60
8/22/47	7/10	70
8/14/47	8/10	80
1/24/49 and 7/1/49	39/39	100

gave 33, 25, 60, 70, and 80 percent deaths. The next test, run after an interval of 17 months, gave 100 percent positive growths. With rare exceptions, C1498 has continued to kill all C57L mice since this date.

Discussion

The *H-2* locus is only one of a number of loci determining susceptibility and resistance to transplants (*1*, *5*). However, it is a particularly "strong" locus, in the sense that where tumor and host differ at this locus, a powerful deterrent against progressive growth is provided. Thus some tumors can successfully surmount the difference between strains BALB/c and DBA/2, C57BL/6 and LP, or C57BR/a and C57BR/cd, which lie within the same *H-2* allelic groups, but with the one exception of tumors tested in strain DBA/1, no tumor can grow consistently across the lines set by the different *H-2* alleles. This is true even of sarcomas 37 and 180, at least when these tumors are inoculated by the methods we used; when double or multiple inoculations are given, these tumors may give nearly 100 percent deaths even in lines not carrying $H\text{-}2^d$.

The fact that specificity with respect to *H-2* alleles is still present in sarcomas 37 and 180 sheds some light on the nature of the changes that occur as tumors acquire the ability to cross strain lines. The presumption is that, in this case at least, the change was in the nature of an increase in virulence rather than a loss of the specificity (antigen?) determined by the original *H-2* genotype (presumed to be $H\text{-}2^d$).

The changes noted in leukemia C1498 tested in C57L hosts are perhaps also most easily explained as due to an increasing virulence. The change must have involved at least two steps, since growth shifted from 0 percent to approximately 50 percent to 100 percent, and may have involved gradual or multiple alterations.

Hauschka and Levan (*6*) have presented evidence indicating a positive relationship in transplantable tumors between tetraploidy and ability to grow in foreign strains. Of the tumors shown in table 1, 6C3HED was found to be diploid, and 15091a, sarcoma 37, and sarcoma 180 to be tetraploid. It would be interesting to determine the ploidy of other tumors in this list. In our tests C1300 and E0771 crossed strain lines at least as easily as 15091a and hence, on the basis of this theory, should be tetraploid.

Summary

The capacity of 13 transplantable tumors to grow in 15 inbred strains of mice has been tested.

As expected, progressive growth occurred in all mice of the native strain.

The growth of transplants in foreign strains (homoiotransplants) was found to be influenced by two factors. 1) The relationship of host and donor strain. Growth was most apt to occur when the two had known, common ancestry. 2) The *H-2* allele carried by host and donor. Growth was most apt to occur when the two had the same *H-2* allele.

Sarcoma 180 grew in 78 of 79 mice of the four strains of the *H-2*^{d} group, but in only 102 of 190 mice of all other *H-2* genotypes. Sarcoma 37 also showed a preference for *H-2*^{d} mice. It is suggested that these two tumors are *H-2*^{d}.

Strain DBA/1 is apparently more susceptible than other strains to tumor homografts.

References

(*1*) LITTLE, C. C.: The genetics of tumor transplantation. *In* The Biology of the Laboratory Mouse (G. D. Snell, ed.). Philadelphia, The Blakiston Co., 1941, pp. 279–309.

(*2*) CARTER, T. C., DUNN, L. C., FALCONER, D. S., GRÜNEBERG, H., HESTON, W. E., and SNELL, G. D.: Standardized nomenclature for inbred strains of mice. Cancer Research 12: 602–613, 1952.

(*3*) BORGES, P. R. F., KVEDAR, B. J., and FOERSTER, G. E.: Development of an isogenic resistant subline of mice (C57BL/6–*H-2*^{d}) from the C57BL/6 strain. In preparation.

(*4*) SNELL, G. D., SMITH, P., and GABRIELSON, F.: Analysis of the histocompatibility-2 locus in the mouse. J. Nat. Cancer Inst. 14: 457–480, 1953.

(*5*) SNELL, G. D.: Methods for the study of histocompatibility genes. J. Genetics 49: 87–108, 1948.

(*6*) HAUSCHKA, T. S., and LEVAN, A.: Inverse relationship between chromosome ploidy and host-specificity of sixteen transplantable tumors. Exper. Cell Research 4: 457–467, 1953.

Influence of Gonadotrophin on Pyridoxine-Deficient and Diet-Restricted Female Mice [1]

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A number of investigators while studying the effects of various deficiencies, most notably those of the vitamin B complex or of thiamine, have observed the result of partial inanition from this cause on the gonads of rats. The work of Marrian and Parkes (*1*), Mason and Wolfe (*2*), Moore and Samuels (*3*), Werner (*4*), and Drill and Burrill (*5*) indicate that in rats partial inanition resulting from vitamin deficiencies decreases hypophyseal secretion, and that hypophyseal insufficiency may be responsible for the gonadal changes noted under such conditions. Mulinos and Pomerantz (*6*) showed that the atrophy and dysfunction of the endocrine organs of rats in prolonged partial inanition closely resembles that following hypophysectomy, and also that injections of chorionic gonadotrophin to underfed rats returned the gonads to a normal state (*7*). Dunn, Morris, and Dubnik (*8*), studying thiamine deficiency in strain C3H mice, found that the histologic changes in the ovaries were paralleled by changes in the organs of pair-fed control mice. However, the interrelationship between partial inanition, nutritional deficiencies, and hormonal insufficiencies as seen in the rat have not been studied in the mouse (*9*). It was, therefore, considered desirable to carry out studies in the mouse which could supply direct experimental evidence to help explain the mechanism whereby dietary restrictions and deficiencies, alone or together, may affect the incidence of spontaneous mammary tumors in mice (*9*).

Severe atrophy of the reproductive organs in female strain DBA mice after prolonged pyridoxine deficiency has been reported by Morris and Dunn (*10*). These initial studies of pyridoxine deficiency in strain DBA mice left it uncertain as to whether the changes in the reproductive organs were caused directly by pyridoxine deficiency or indirectly through a failure of the pituitary to produce or release a gonad-stimulating hormone. It was thought that pyridoxine might be essential for the synthesis of the protein-containing, gonad-stimulating hormone of the pituitary.

A brief account of an extensive study made on the hematologic response

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of pyridoxine-deficient and gonadotrophin-stimulated mice is presented. Some spontaneous lesions peculiar to DBA mice have also been noted.

Methods

Weanling DBA/2 female mice [3] approximately one month of age were selected at random and divided into 8 groups. Although 10 to 13 animals were originally included in each group, the final number varied from 3 to 13, because animals found dead were not autopsied and survival in the different groups varied.

The groups and their designations were as follows:

Group 1, B_6-2.5, G-O.—The symbol B_6-2.5 indicates that 2.5 mg. of pyridoxine was added to each kilogram of the synthetic diet, which provides an adequate amount of pyridoxine for animals whose food intake is restricted. G-O indicates that no gonadotrophin was given. Ten mice in Group 1 were autopsied after an average total period of 84 days.

Group 2, B_6-2.5, G-S.—G-S indicates that gonadotrophin injections were given for an average of 23 days (short period) beginning after the mice had been 7 weeks on the diet. Seven mice were autopsied after an average total period of 72 days.

Group 3, B_6-2.5, G-L.—G-L indicates that gonadotrophin injections were begun 2 weeks after the mice were put on the diet, and injections were continued for an average of 67 days (long period). Seven mice were autopsied after an average total period of 81 days.

Group 4, B_6-0, G-O.—B_6-0 indicates a pyridoxine-deficient diet. Three mice were autopsied after an average total period of 77 days.

Group 5, B_6-0, G-S.—Indicates that a pyridoxine-deficient diet was given for 7 weeks, then injections with gonadotrophin were made for an average of 19 days (short period). Three mice were autopsied after an average total period of 68 days.

Group 6, B_6-0, G-L.—Indicates that gonadotrophin injections were begun 2 weeks after starting the deficient diet, and continued for an average of 53 days (long period). Three mice were autopsied after an average period of 67 days.

Group 7, R_4, B_6-2.5, G-O.—R_4 indicates mice whose food intake was restricted to the amount consumed by pair-mates in Group 4. Nine mice were autopsied after the same time interval as the pyridoxine-deficient member of the pair, an average of 77 days.

Group 8, R_5, B_6-2.5, G-S.—R_5 indicates mice whose food intake was restricted to the amount consumed by pair-mates in Group 5. Eight mice were autopsied after the same time interval as the pyridoxine-deficient member of the pair, an average of 68 days.

The animals in the pyridoxine-deficient groups were fed the following diet in grams: vitamin-free casein 180 (Labco brand), cerelose 570, salt mixture 45 (*11*), lard 170, lard 10 (containing 10,000 vitamin A units as vitamin A-ester and 100 mg. α-tocopherol acetate), choline 5, and cod-liver oil 20. A pyridoxine-free vitamin mixture consisting of the following in mg. per kg. of diet: folacin 10, *d*-biotin 1, thiamine·HCl 40, ribo-

[3] Obtained from S. M. Polley, National Institutes of Health.

flavin 20, niacin 200, and calcium pantothenate 140, was thoroughly mixed with a 30-gm. portion of the cerelose and added to the diet.

Each animal in the groups selected for gonadotrophin treatment received daily 5 International Units of pregnant mare serum gonadotrophin [4] in physiological saline. A 0.25-ml. dose of gonadotrophin was injected daily in the thigh muscles of the right and left hind legs, alternately.

The mice in the groups receiving adequate pyridoxine were fed the above diet, to which 2.5 mg. of pyridoxine·HCl per kg. of diet was added. The pyridoxine was thoroughly mixed in a portion of the cerelose before it was added to the diet mixture.

All mice were kept in individual cages with wire-cloth bottoms. Water was available at all times. Animals were weighed weekly and weekly records of food intake were maintained. Each mouse in the restricted-diet groups (7 and 8) was given daily ½ the amount of food eaten on an *ad libitum* basis during the previous week by its pair-mate in Groups 4 and 5, respectively.

Autopsies were done under magnification with the dissecting microscope. The following organs were weighed with the Roller-Smith torsion balance as soon as they were removed: spleen, left kidney, both ovaries together, uterus with attached Fallopian tubes, and the heart after all chambers had been opened and the blood gently blotted away. The following organs or portions of them were routinely taken for microscopic study: eye, heart, lung, thymus gland, stomach, duodenum just below the pylorus, mid-portion of the ileum, straight portion of the colon above the anus, spleen, liver, kidney, ovary, uterus, vagina, adrenal gland, mesenteric lymph node and some peripheral lymph nodes (usually the cervical and inguinal), salivary gland, thyroid gland, mammary gland tissue, skin (occasionally), sternum with bone marrow, brain, and pituitary gland. All sections except the pituitary gland and the sternum were routinely fixed with Zenker's formol, embedded in paraffin, and stained with hematoxylin and eosin and by the prussian-blue method. The same procedure was carried out with the sternum after decalcification. Selected sections of the kidney were later treated by the periodic acid-Schiff method. The pituitary gland was removed, fixed in Bouin's fluid, and stained by the aldehyde-fuchsin method of Gomori. The hematologic studies in these animals consisted of red and white cell counts, differential counts, hemoglobin determinations, and a few reticulocyte counts (*12*).

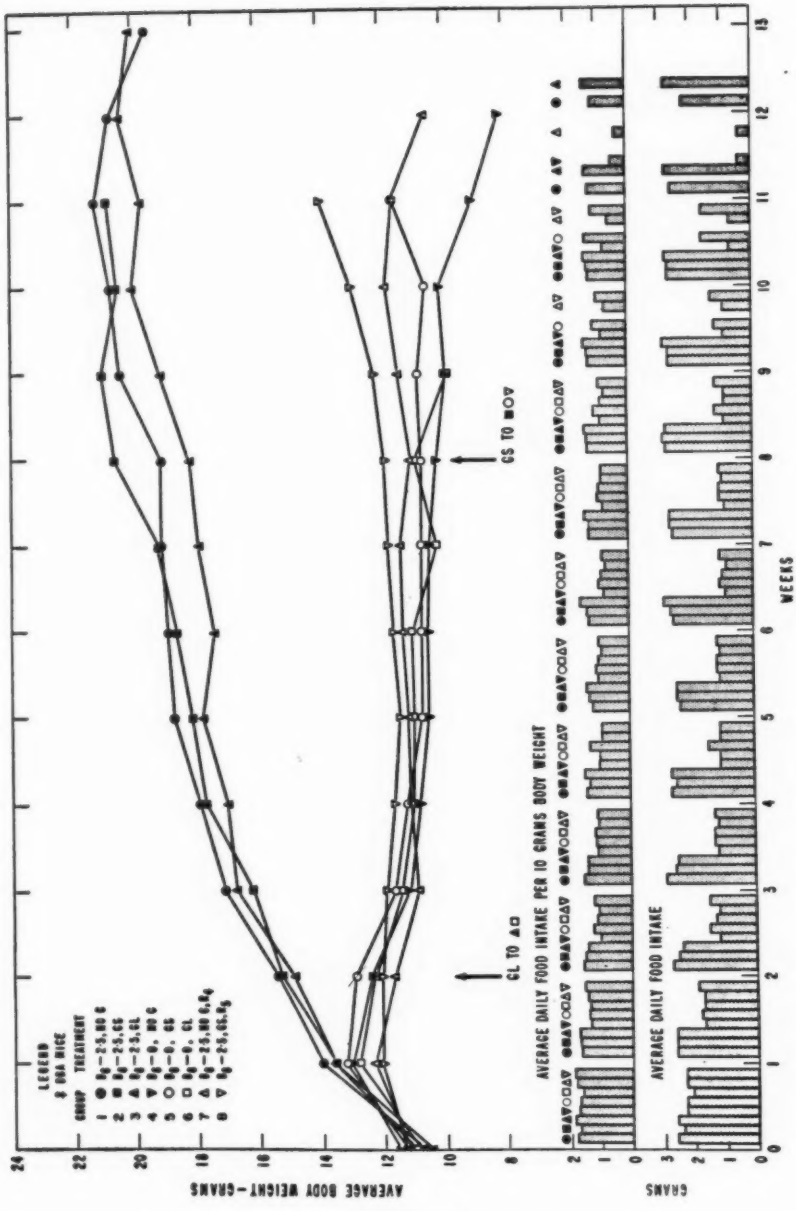
Results

The results are reported under five headings: 1) Body weight and food consumption, 2) reproductive and endocrine organs, 3) other organs affected by gonadotrophin injections, 4) hematologic findings, and 5) lesions of strain DBA mice.

1) Body weight and food consumption

Curves showing the average body weights for each of the eight groups are presented in text-figure 1. The mice in all the groups receiving food *ad libitum* supplemented with adequate pyridoxine (Groups 1, 2, and 3) increased uniformly in weight in a manner suggesting the typical normal weight response of DBA mice to this diet. The pyridoxine-deficient and the diet-restricted groups, after a first-week gain, decreased slowly in weight until they reached approximately their average initial weight, which was then maintained fairly constant in all five groups for the rest of the experimental period. Weight values for each group after the tenth week cannot be considered significant since they represent averages for too few mice.

[4] Anteron, obtained from Schering Corp., Bloomfield, N. J.



TEXT-FIGURE 1.—Average body weights, average daily food intake, and average daily food intake per 10 gm. of body weight, for mice in 8 groups as indicated.

The average daily food intake per mouse at weekly intervals for each group (after correcting for spilled food) are shown as bar graphs at the bottom of text-figure 1. The food intake for the three pyridoxine-deficient groups (4, 5, and 6) decreased sharply after the first week to values somewhat less than half those for Groups 1, 2, and 3. The average food intake and body weight of the pyridoxine-deficient and diet-restricted groups paralleled each other as shown in text-figure 1.

The food intake values per each 10 grams of average body weight (the upper series of bar graphs in text-fig. 1) are only slightly less for the deficient and restricted mice per unit of weight than for the pyridoxine-supplemented animals.

The effect of gonadotrophic hormone injections on either food intake or body weight was negligible.

2) Reproductive and endocrine organs

The histologic changes in the ovary, vaginal epithelium, uterus, mammary gland, and adrenal gland are listed in table 1. The average weights of the ovaries and uterus, expressed as percent of body weight, are shown in text-figure 2.

Ovaries.—The weight of the ovary in the different groups was altered both actually and in proportion to body weight (text-fig. 2). It generally increased after the injection of gonadotrophin, both in the pyridoxine-deficient and the diet-restricted animals. The largest increase occurred in Group 5 (B_6-O, G-S) mice. The average ovary weight in proportion to body weight in this group was increased by a factor of 10. The weight was much above the weight of the ovaries in Group 6 (B_6-O, G-L) mice. The weights were also greater than the ovaries in Group 8 (R_5, B_6-2.5, G-S) mice. Much less, if any, weight response to gonadotrophin injections was observed in the pyridoxine-supplemented animals.

Ovaries found in all mice in the different groups were generally uniform for the group, though cyclic changes caused normal variations in the pyridoxine-supplemented mice. A general histologic type could be described (table 1) and representative photographs for each group are shown in figures 1 to 8.

Vaginal epithelium.—Changes in the vaginal epithelium were generally consistent with the alterations noted in the ovaries (table 1).

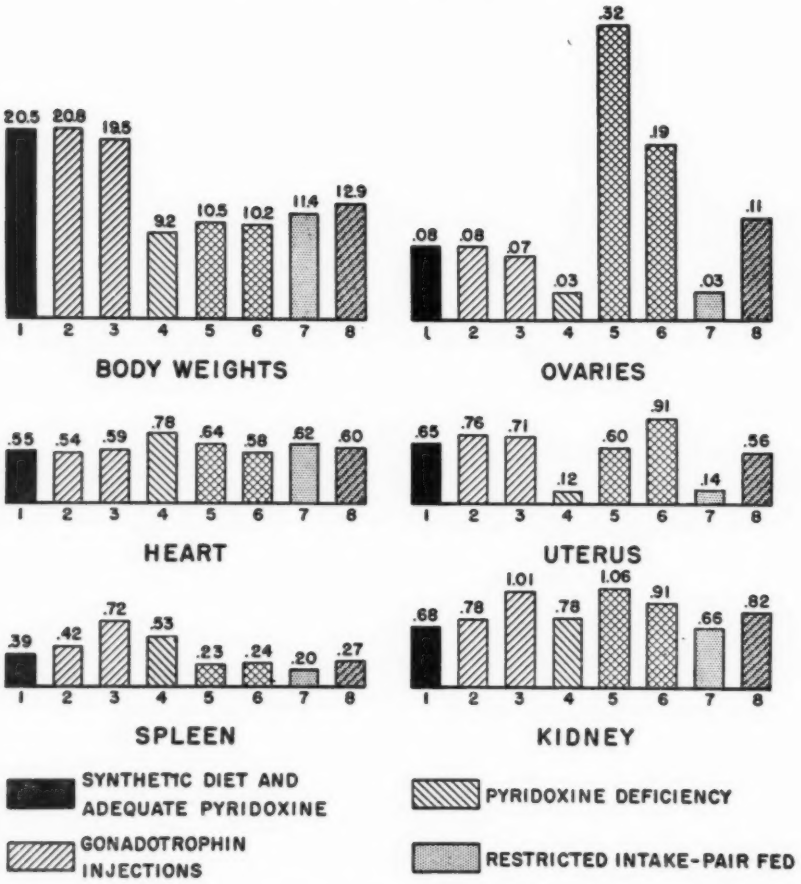
Uterus.—This organ also showed variations consistent with the alterations noted in the ovaries (figs. 9 and 10, and table 1). A notable change was observed in the endometrial stroma of the mice treated with gonadotrophin (fig. 10). A considerable amount of intercellular amorphous hyaline substance was found, and it seemed doubtful whether this change was reversible. Extreme atrophy of the uterus was found in the pyridoxine-deficient and the pair-fed mice without gonadotrophin.

Mammary gland tissue.—The histologic changes are given in table 1. Secretory activity and a considerable proliferation of acini were noted

TABLE 1.—*Histologic changes in reproductive organs and adrenal gland*

Group	Ovary	Mammary gland	Uterus	Vaginal epithelium	Adrenal gland
1 B_6-2.5 G-O (Normal)	Normal. Follicles in various stages of development. Well-formed corpora lutea. Moderate number of atretic follicles.	Mammary ducts observed. Variation with cycle.	Variable, depending on stage of estrous cycle.	Variable, depending on stage of estrous cycle.	Degenerating X zone with varying amount of lipoid tissue.
2 B_6-2.5 G-S	Well-formed corpora lutea. Occasional cystic follicles, some hemorrhagic.	Piling up of epithelium in ducts. Generally no acinar proliferation.	Columnar epithelium. Edematous stroma. Variable with cycle.	Somewhat variable, depending on cycle.	Lipoid degeneration of X zone in many.
3 B_6-2.5 G-L	Some normal follicles. Cystic follicles never large, occasionally hemorrhagic. Notable number of vacuolated interstitial cells. Some "ceroid" cells.	Ducts dilated. Acinar secretion and proliferation sometimes extreme.	Columnar epithelium; sometimes extreme vacuolation of the surface cells. Hyalinized stroma.	No cornification. Increase in thickness of mucosa. Sometimes extreme vacuolation of the surface cells.	Complete degeneration of X zone. Generally no lipoid cells.
4 B_6-O G-O	Abundant ova. Small to medium-sized follicles. No luteal tissue. Shrunken interstitial cells. Many atretic follicles.	Not observed.	Extreme atrophy. Low cuboidal epithelium.	Extreme atrophy, only 2 cell-layers thick.	Little or no X-zone degeneration. No lipoid cells.
5 B_6-O G-S	Numerous very large cysts. Abundant luteal tissue, no formed corpora lutea. Relatively few ova and small follicles.	Dilated ducts and piling up of epithelium.	High columnar epithelium. Hyalinized stroma.	Increase in thickness. Surface cells often rounded, vacuolated, and desquamated.	X zone usually completely degenerated. No lipoid.
6 B_6-O G-L	Abundant luteal tissue but no well-formed corpora lutea. A few large cystic follicles and hemorrhagic cysts.	Piling up of epithelium in ducts. No acinar proliferation. Slight secretion in ducts.	Columnar epithelium. Some cells vacuolated and necrotic. Hyalinized stroma, cystic cavity.	"	"

7 R_4 B_6-2.5 G-O	Similar to Group 4, except for slightly vacuolated cells in medulla.	Only a few ducts observed. Appear atrophic.	Similar to Group 4.	Similar to Group 4, but changes less severe.	X zone partly degenerated. No lipoid.
8 R_5 B_6-2.5 G-S	No very large cysts, some hemorrhagic. No discrete well-formed corpora lutea. Resemble ovaries in Group 3.	Proliferating mammary-duct epithelium. No acinar proliferation.	Columnar epithelium. Moderately hyalinized stroma.	Increase in thickness. Extreme vacuolation of surface cells.	Degenerated X zone. No lipoid.



TEXT-FIGURE 2.—Average weights of organs expressed as percent of body weight in female DBA mice of 8 groups.

in Group 3 (B_6-2.5, G-L). No other groups showed any considerable acinar proliferation, a finding which suggests that both sufficient stimulation from gonadotrophin and an adequate food intake were required. Proliferation of duct epithelium was noted in the deficient mice given gonadotrophin, and the pyridoxine-supplemented mice receiving gonadotrophin for a short period.

Adrenal gland.—The adrenal changes were generally uniform for each group of mice. The appearance of the X zone was the most distinctive feature in each group (table 1). In Group 1 (B_6-2.5, G-O) the X zone was being replaced by a wide zone of lipoid cells, an alteration during degeneration of the X zone which often appears in the mouse (fig. 11). Gonadotrophin injections appeared to hasten the degeneration of the X zone. In Group 4 (B_6-O, G-O) nearly complete preservation of the X

zone was observed (fig. 12). The X zone was partially or completely degenerated in pyridoxine-deficient, gonadotrophin-treated animals (fig. 13), and in most pair-fed animals there were no lipoid cells.

3) Other organs affected by gonadotrophin injections

Kidney.—Inspection of text-figure 2 shows that enlargement of the kidney accompanied gonadotrophin injections in all groups of mice. Histologically, the enlarged kidneys showed no striking alteration and the enlargement appeared to be caused by a general hyperplasia of all portions of the kidney.

Skin.—A dermatitis in an area anterior to the vaginal opening was recorded in animals in Group 3 (B_6-2.5, G-L). Depilation followed by desquamation of the surface epithelium left a weeping, raw surface. Histologically, a hyaline necrotic membrane appeared on the surface and the subcutaneous tissue was edematous, with inflammatory-cell infiltration. Abscesses were noted in a few animals (fig. 14). Skin lesions of this type were not observed regularly in any other group.

Sections of the duodenum and ileum occasionally appeared edematous in Group 3 (B_6-2.5, G-L) but this finding was infrequent and could not be directly ascribed to the treatment.

4) Hematologic findings

The results of this part of the experiment gave no new clues as to the cause of the anemia in pyridoxine deficiency. The results of the leukocyte counts (expressed separately for the granulocytes and lymphocytes), the erythrocyte counts, the hemoglobin, and the distribution of iron-positive substance in various organs was arranged in a table. From inspection of this table, the following general results can be reported:

No significant change developed when gonadotrophin was given for a short period. An increase in granulocytes, and a decrease in erythrocytes and hemoglobin appeared in Group 3 (B_6-2.5, G-L) mice, but this change was probably secondary to inflammatory reactions appearing particularly in the skin in these animals.

Clinical symptoms of anemia such as weakness and pallor developed acutely in mice of Group 4 (B_6-0, G-O) shortly before death. An extreme reduction in lymphocytes was found, and a considerable increase in granulocytes. The erythrocyte and hemoglobin values were sometimes so low as to make accurate estimation uncertain (average 1.83 million erythrocytes, 1.86 gm. hemoglobin). Hemoglobin determinations and erythrocyte counts made on 3 mice after 7 weeks of pyridoxine deficiency were within the normal range. Pyridoxine-deficient mice receiving gonadotrophin survived for a shorter period, and did not develop a severe terminal anemia.

Observation of the fresh blood with the phase microscope and study of the stained smears showed significant changes in Group 4 (B_6-0, G-O). Anisocytosis and basophilia of the erythrocytes was extreme. Especially notable were large, flattened, platelike erythrocytes, often showing only a narrow rim of stained material. The centers of these erythrocytes were pale, or might contain a small mass of stained substance (target cells). Under the phase microscope, or when a stain (cresyl violet) for reticulocytes was used on the smear, these platelike cells were seen to contain granules at the margins, and they were identified as reticulocytes. "Crescents" and "ghost" cells were also frequent in the smears.

Iron-positive substance was found in the epithelial cells of the duodenum and colon in both control and treated mice, and could not be correlated with any experimental procedure. The material appeared in granular form on the luminal side of the nucleus

and as a diffuse faint blue in the basal portion of the cell. This difference on either side of the nucleus has been previously noted by Cappell (*13*) and Endicott *et al.* (*14*).

The amount of iron appeared to be significantly increased in the liver of mice of Group 4 (B_6-O, G-O). It was absent from the hepatic cells in all other groups except for minute quantities in some of the pair-fed animals. The presence of iron-positive material in other organs was not considered significant. The absence of iron-positive material in the kidney in all groups was noted. No iron was observed in the mammary tissue, which Rawlinson (*15*) has usually found in older mice.

Autopsy findings and histologic examination of the hematoxylin and eosin preparations of organs active in blood formation or destruction were not significant as regards the cause of the anemia in pyridoxine deficiency, except that evidence of active erythropoiesis was found in the spleen and bone marrow in some severely deficient mice. No direct effect of the gonadotrophin injections was observed on the blood-forming organs.

5) Lesions of strain DBA mice

The thyroid gland, pituitary gland, lung, brain, gastrointestinal tract, and liver showed no significant lesions related to the experimental procedure, except for the increased deposition of iron previously described in the liver.

Several lesions found in both control and treated animals are probably peculiar to strain DBA mice, and are not related to the experimental procedure. These were degenerative lesions in the myocardium, the skeletal muscle, the subcutaneous fat, and the cornea. The lesions varied in degree in the myocardium (fig. 15) and skeletal muscle, but typically showed a central area of necrosis, strongly fuchsinophilic on periodic acid-Schiff treatment, with large mononuclear cells adjacent to and sometimes surrounding the necrotic material. This necrotic material sometimes appeared basophilic with hematoxylin and eosin, and suggested that calcification was developing.

The corneal lesion (fig. 16) appeared in microscopic sections like a long thin needle of basophilic homogeneous material lying in a narrow space, probably produced by shrinkage during fixation. Some large cells were seen at the margin of the space. On examination of the living mouse, irregular white opaque patches were sometimes observed in the central portion of the cornea. These patches probably corresponded to the microscopic lesion seen in cross section.

Discussion

Gonadotrophic hormone injections in pyridoxine-deficient and diet-restricted female mice of strain DBA indicate ability of the end organ (the ovary) to respond, suggesting that hypophyseal insufficiency may be responsible for the gonadal changes. The mouse thus appears to respond in a manner similar to the rat during prolonged partial inanition. Effects of pyridoxine deficiency may be similar to those produced by partial inanition.

Thus, the failure to observe mammary gland tissue in microscopic sections in pyridoxine-deficient animals and the presence of a few glands in diet-restricted animals suggests that a somewhat greater effect on the mamma occurred in the double deficiency than occurred in animals restricted only in food. Regardless of how produced, a prolonged insufficiency of gonadotrophic hormone that inhibits the development of the mammary glands, points strongly to the probable role of the hypophysis in spontaneous mammary tumor development (*9*). The use of a strain of mice that develops spontaneous mammary tumors, such as the one used in these experiments, may make possible the experimental demonstration

of such an insufficiency upon the development of spontaneous mammary tumors. However, such a demonstration may be extremely difficult to obtain because of the difficulties occurring in experimental animals subjected to prolonged administration of an exogenous protein-containing gonadotrophin. The preparation and use of a very pure gonadotrophin might obviate these difficulties. The problem certainly merits further study.

The histologic changes in the endocrine and reproductive organs were generally consistent with the alterations in the ovary. Well-formed discrete corpora lutea did not appear in the pyridoxine-deficient mice. The difference in response in the pyridoxine-supplemented controls and the pyridoxine-deficient animals was noteworthy, since a more extreme response appeared in the pyridoxine-deficient mice given gonadotrophin for the shorter period of time. It would appear that the presence of a more nearly normal endocrine system in a supplemented animal prevented the extreme reaction to gonadotrophin found in the deficient mice. The hyaline appearance of the endometrial stroma was directly related to the amount of gonadotrophin administered, and it seems doubtful whether this change was reversible.

The relative increase in the size of the kidney following gonadotrophin injections was observed in all groups of mice. The kidney is said to increase in size after treatment with certain hormones (*16*).

An inflammatory lesion of the skin on the ventral surface adjacent to the vagina was regularly found in normally fed animals receiving prolonged injections of gonadotrophin and appeared to be directly related to treatment. An increase in granulocytes often accompanied the skin reaction. No previous report concerning such an effect of gonadotrophin has been found.

The granulocytosis and lymphopenia developing during pyridoxine deficiency were in agreement with the results of Weir, Heinle, and Welch (*17*). The hematologic response to pyridoxine deficiency has been reported to vary with different species (*18*), and the cause of the anemia has not been determined. In the present study, no positive iron staining developed in the kidney, such as is usually found when there is an excessive hemoglobin destruction or when iron compounds are injected in the mouse (*13*). No noticeable evidence of blood destruction was detected in other tissues. The anemia did not appear to be aplastic in type, since active erythropoiesis was found in severely deficient animals. The peculiar morphology of many reticulocytes as revealed by the phase microscope and in smears stained to demonstrate reticulocytes was noteworthy. Thin, hypochromic erythrocytes and "target" cells are not considered pathognomonic of any condition, but they have been reported in iron-deficiency anemia and during posthemorrhagic erythropoiesis (*19*). A considerable accumulation of iron in the hepatic cells in pyridoxine-deficient mice suggested a failure in the metabolism of iron, although the prussian-blue reaction in the duodenum and colon did not differ greatly in the pyridoxine-deficient and control animals. The iron staining of

epithelial cells in the mucosa of the colon, similar to that in the duodenum, was unexpected since no previous description has been found of stainable iron in the colon in experimental mice. Stainable iron was expected in the duodenum, the site from which it is reportedly absorbed (*20*).

Reports of the effects of various hormones on blood formation and destruction (*21*) and the often unexplained anemia of pregnancy (*22*) prompted the present investigation of the possible effect of gonadotrophin on the blood of the mouse. The results were essentially negative.

Other lesions such as focal necrosis of the myocardium and skeletal muscle, and degenerative lesions in the cornea, were probably spontaneous lesions in strain DBA mice and not related to the experimental procedure.

Summary

1) Eight groups of weanling strain DBA female mice were used to study the changes resulting from prolonged gonadotrophin injection under conditions of pyridoxine deficiency and diet restriction. Gonadotrophin was injected beginning after the 2nd or 7th experimental week for variable periods ranging from 3 to 10 weeks.

2) The pyridoxine-deficient and the diet-restricted mice showed no net gain in body weight, while pyridoxine-supplemented controls gained normally. Gonadotrophin injections had little effect on average food consumption or average body weights. The weight curves reflect the food intake for each group, but the differences in food consumption become much less significant when expressed in terms of intake per unit weight.

3) The ovaries of the pyridoxine-deficient and the diet-restricted mice were histologically similar. Gonadotrophin injections greatly increased the size of the ovaries. A greater increase occurred in the size of the ovaries of deficient mice than in the ovaries of pyridoxine-supplemented animals.

4) The mammary gland tissue in both the deficient and supplemented mice treated with gonadotrophin showed proliferation of the duct epithelium. The mammary tissue in supplemented mice given gonadotrophin over a long period showed secretory activity and acinar proliferation.

5) The histologic changes noted in vaginal and uterine epithelium were consistent with the condition of the ovaries. The uterus of gonadotrophin-injected animals showed a hyaline intercellular substance in the endometrial stroma, a change which was probably irreversible.

6) Pyridoxine deficiency prevented lipoid degeneration of the X zone of the adrenal gland, while only partial degeneration, with no lipoid cells, occurred in the diet-restricted animals. Gonadotrophin caused the disappearance of the X zone in the deficient animals, without lipoid formation.

7) A severe anemia developed acutely after prolonged pyridoxine deficiency. It was characterized by extremely low hemoglobin values and erythrocyte counts, and large, platelike reticulocytes of unusual morphology. The lymphocyte count was greatly reduced. Gonadotrophin had no direct effect on the blood picture.

References

(*1*) MARRIAN, G. F., and PARKES, A. S.: The effect of anterior pituitary preparations administered during dietary anoestrus. Proc. Roy. Soc., London, s. B. 105: 248–258, 1926.

(*2*) MASON, K. E., and WOLFE, J. M.: The physiological activity of the hypophyses of rats under various experimental conditions. Anat. Rec. 45: 232, 1930.

(*3*) MOORE, CARL R., and SAMUELS, L. T.: The action of testis hormone in correcting changes induced in the rat prostate and seminal vesicles by vitamine B deficiency or partial inanition. Am. J. Physiol. 96: 278–288, 1951.

(*4*) WERNER, S. C.: Failure of gonadotropic function of the rat hypophysis during chronic inanition. Proc. Soc. Exper. Biol. & Med. 41: 101–105, 1939.

(*5*) DRILL, V. A., and BURRILL, M. W.: Effect of thiamin deficiency and controlled inanition on ovarian function. Endocrinology 35: 187–192, 1944.

(*6*) MULINOS, M. G., and POMERANTZ, L.: Pseudo-hypophysectomy. A condition resembling hypophysectomy produced by malnutrition. J. Nutrition 19: 493–504, 1940.

(*7*) ———The reproductive organs in malnutrition. Effects of chorionic gonadotrophin upon atrophic genitalia of underfed male rats. Endocrinology 29: 267–275, 1941.

(*8*) DUNN, T. B., MORRIS, H. P., and DUBNIK, C. S.: Lesions of chronic thiamine deficiency in mice. J. Nat. Cancer Inst. 8: 139–155, 1947.

(*9*) MORRIS, H. P.: Some nutritional factors influencing the origin and development of cancer. J. Nat. Cancer Inst. 6: 1–17, 1945.

(*10*) MORRIS, H. P., and DUNN, T. B.: Pyridoxine deficiency—nutritional and histological observations on five strains of mice. 12th Congress of Pure and Applied Chemistry, N. Y., Sept. 10–13: 164–165, 1951.

(*11*) MORRIS, H. P., and ROBERTSON, W. v. B.: Growth rate and number of spontaneous mammary carcinomas and riboflavin concentration of liver, muscle, and tumor of C3H mice as influenced by dietary riboflavin. J. Nat. Cancer Inst. 3: 479–489, 1943.

(*12*) BATEMAN, J. C.: Leukemoid reactions to transplanted mouse tumors. J. Nat. Cancer Inst. 11: 671–687, 1951.

(*13*) CAPPELL, D. F.: The late results of intravenous injection of collodial iron. J. Path. & Bact. 33: 175–196, 1930.

(*14*) ENDICOTT, K. M., GILLMAN, T., BRECHER, G., NESS, A. T., CLARKE, F. A., and ADAMIK, E. R.: A study of histochemical iron using tracer methods. J. Lab. & Clin. Med. 34: 414–421, 1949.

(*15*) RAWLINSON, H. E.: The use of an iron stain for the study of alveolar development in the mouse mammary gland. Canad. J. Res., Sect. E, 28: 1–4, 1950.

(*16*) PFEIFFER, C. A., EMMEL, V. M., and GARDNER, W. U.: Renal hypertrophy in mice receiving estrogens and androgens. Yale J. Biol. & Med. 12: 493–501, 1940.

(*17*) WEIR, D. R., HEINLE, R. W., and WELCH, A. D.: Role of pyridoxine in the production of leucocytes in normal and leukemic mice. Proc. Soc. Exper. Biol. & Med. 72: 457–461, 1949.

(*18*) FOLLIS, R. H.: The Pathology of Nutritional Disease; Physiological and Morphological Changes which Result from Deficiencies of the Essential Elements, Amino Acids, Vitamins, and Fatty Acids. Springfield, Ill., Charles C. Thomas, 1948.

(*19*) CROSBY, W. H.: The pathogenesis of spherocytes and leptocytes (target cells). Blood 7: 261–274, 1952.

(*20*) GRANICK, S.: Iron metabolism and hemochromatosis. Bull. New York Acad. Med. 25: 403–428, 1949.

(*21*) CRAFTS, R. C.: The effects of estrogens on the bone marrow of adult female dogs. Blood 3: 276–285, 1948.

(*22*) TYSOE, F. W., and LOWENSTEIN, L.: Blood volume and hematologic studies in pregnancy and the puerperium. Am. J. Obst. & Gynec. 60: 1187–1205, 1950.

PLATE 35

Ovaries from representative mice from each of 8 groups. Hematoxylin and eosin. X 40

FIGURE 1.—Normal ovary (from Group 1) showing well developed follicles and numerous discrete corpora lutea. Group 1 (B_6-2.5, G-O).

FIGURE 2.—Ovary from pyridoxine-supplemented mouse after short period of gonadotrophin injections. Two cystic follicles with hemorrhage. Group 2 (B_6-2.5, G-S).

FIGURE 3.—Ovary from pyridoxine-supplemented mouse after long period of gonadotrophin injections. Medulla occupied by conglomerate masses of luteal tissues. A few small hemorrhagic cysts. Group 3 (B_6-2.5, G-L).

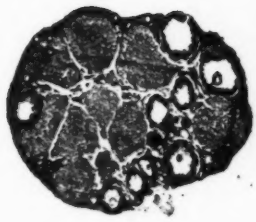
FIGURE 4.—Ovary from pyridoxine-deficient mouse. Severe atrophy, many small and medium-sized follicles, no corpora lutea, many atretic follicles. Group 4 (B_6-0, G-O).

FIGURE 5.—Ovary from pyridoxine-deficient mouse receiving gonadotrophin injections for a short time. Numerous large cysts, some luteal tissue, but no formed corpora lutea. Group 5 (B_6-0, G-S).

FIGURE 6.—Ovary from pyridoxine-deficient mouse receiving gonadotrophin injections for a long time. Many cystic follicles, lined by greatly vacuolated cells. Group 6 (B_6-0, G-L).

FIGURE 7.—Ovary from mouse pair-fed to Group 4. Ovary very small, many small and medium-sized follicles, no corpora lutea, many atretic follicles. Group 7 (R_4, B_6-2.5, G-O).

FIGURE 8.—Ovary from mouse pair-fed to Group 5. Many cystic follicles, some of which are hemorrhagic. No well-formed discrete corpora lutea. Group 8 (R_5, B_6-2.5, G-S).



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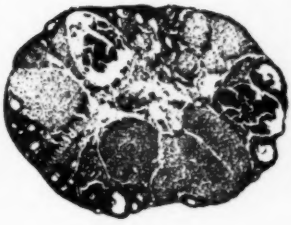

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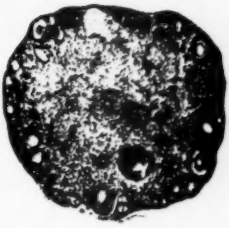

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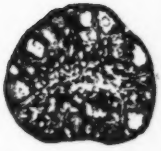

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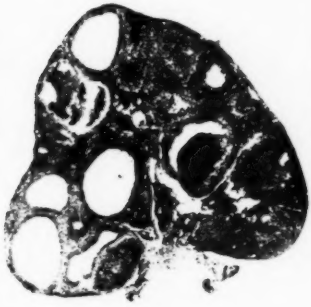

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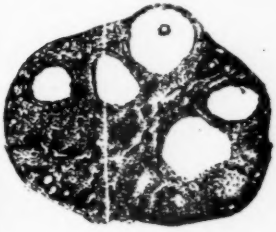

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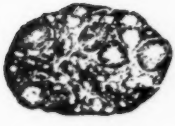

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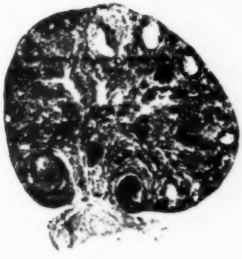

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273201—53——5

PLATE 36

FIGURE 9.—Endometrium. Group 1 (B_6-2.5, G-O). Adequate pyridoxine, no gonadotrophin. Endometrium at estrus. Note cellular edematous-appearing stroma, and columnar epithelium. Hematoxylin and eosin. × 250

FIGURE 10.—Endometrium. Group 3 (B_6-2.5, G-L). Adequate pyridoxine, gonadotrophin for long period. Note hyaline intercellular material in stroma, and high columnar epithelium. Hematoxylin and eosin. × 250

FIGURE 11.—Adrenal cortex. Group 1 (B_6-2.5, G-O). Adequate pyridoxine, no gonadotrophin. Note degeneration of X zone and layer of lipoid cells next to the medulla. Hematoxylin and eosin. × 250

FIGURE 12.—Adrenal cortex. Group 4 (B_6-O, G-O). Pyridoxine-deficient, no gonadotrophin. X zone present between cortex and medulla. Hematoxylin and eosin. × 250

FIGURE 13.—Adrenal cortex. Group 6 (B_6-O, G-L). Pyridoxine-deficient, gonadotrophin for long period. Note degeneration of X zone and presence of amorphous eosinophilic material in this region. Hematoxylin and eosin. × 250

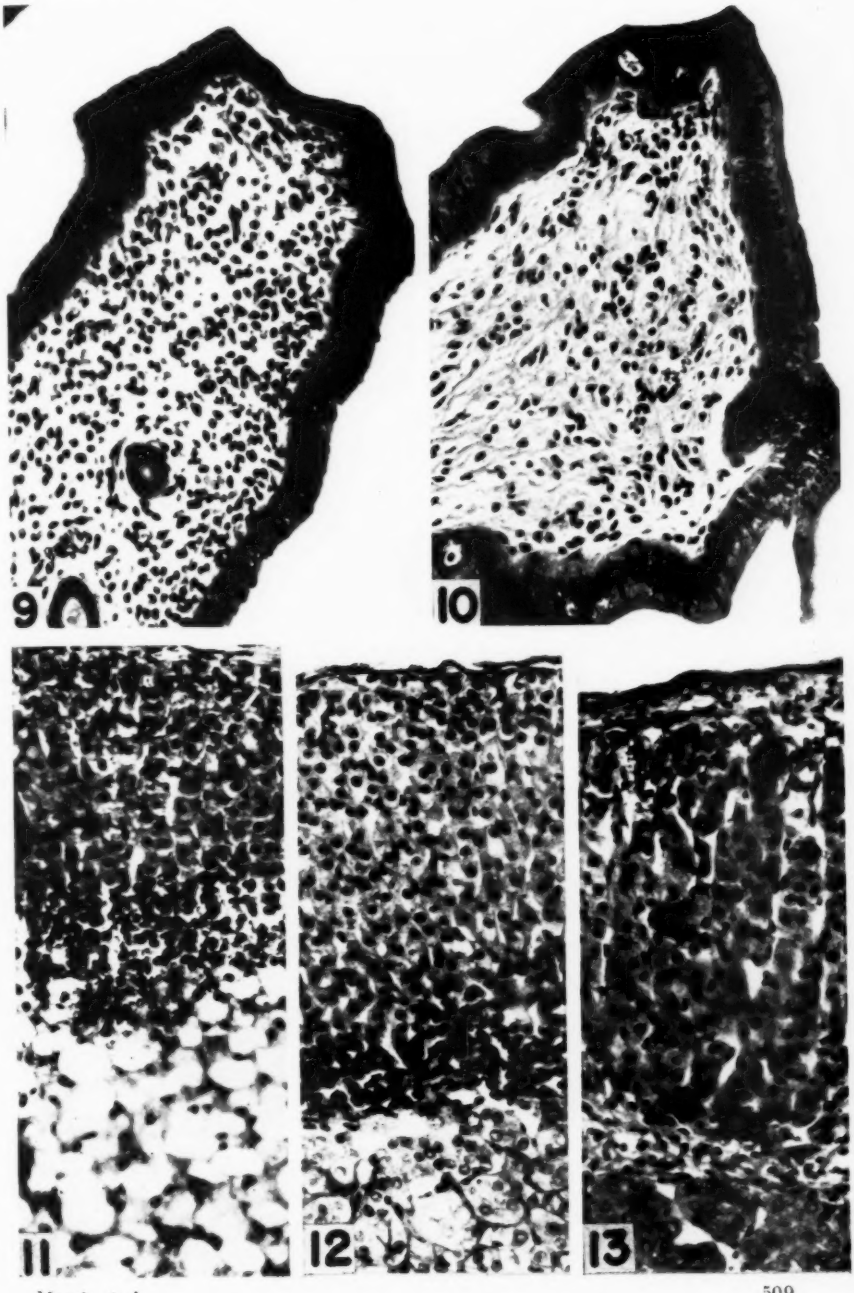
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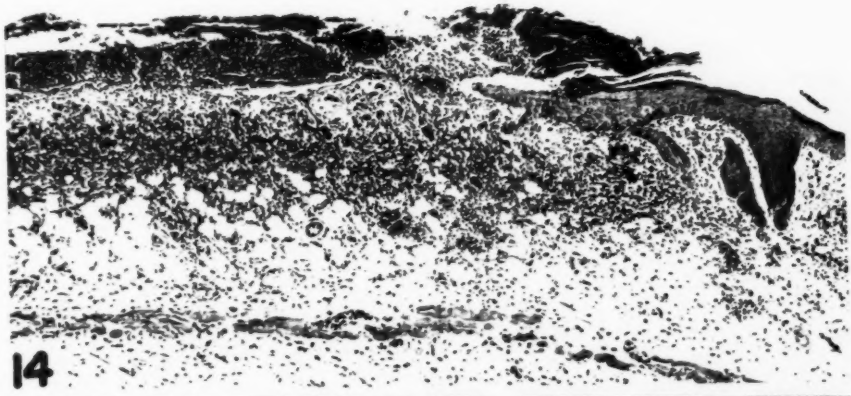
PLATE 37

FIGURE 14.—Section from skin near vagina. Group 3 (B_6-2.5, G-L). Adequate pyridoxine, gonadotrophin for long period. Note loss of epidermis, inflammatory pseudomembrane, inflammatory reaction in dermis with granulation tissue, and leukocytic infiltration. Hematoxylin and eosin. × 80

FIGURE 15.—Section in myocardium. Group 1 (B_6-2.5, G-O). Adequate pyridoxine, no gonadotrophin. Area of necrosis in myocardium, surrounded by a capsule of spindle cells, some with very large nuclei. Hematoxylin and eosin. × 290

FIGURE 16.—Lesion in cornea. Group 1 (B_6-2.5, G-O). Adequate pyridoxine, no gonadotrophin. Lesions in cornea consisting of thin sheets of amorphous material with some fibroblastic reaction and vascularization at the margin. Hematoxylin and eosin. × 290



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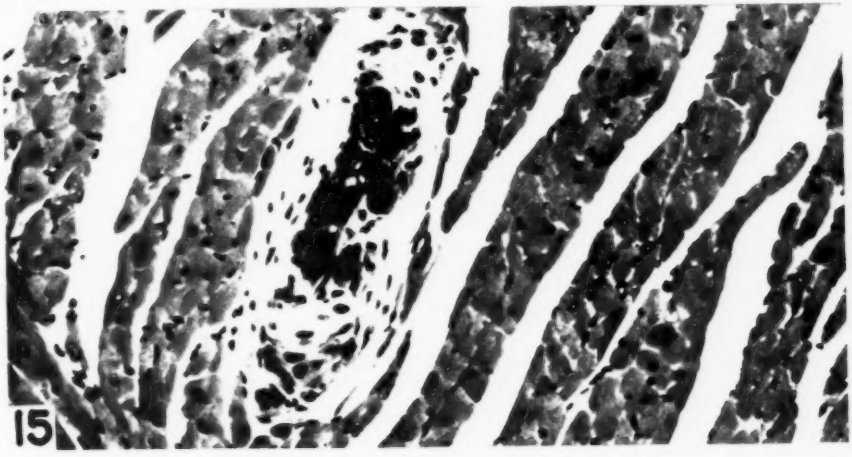

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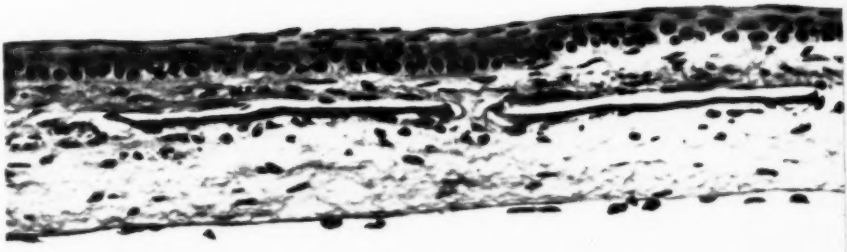

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273201—53——6

The Effects of Amino Acids as Supplements to Fractions of Serum and Embryo Extract on the Proliferation of Cells *in Vitro* [1]

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In an earlier study (*1*) [3] protein-free ultrafiltrates and protein-containing residues [4] of both chick-embryo extract and of horse serum were tested on a strain of mouse cells of single-cell origin to establish the groups of components actually used by the cells from a serum–embryo extract culture fluid. The practical objective was to determine whether any fraction of either horse serum or chick-embryo extract could be eliminated from the culture fluid without affecting cell proliferation and growth. The elimination of any unnecessary fraction might simplify chemical analyses of the medium and thus facilitate the ultimate synthesis of a chemically defined culture fluid. The most significant outcome of this study was the finding that cultures grown in a fluid mixture of chick-embryo-extract ultrafiltrate and dilute horse serum attained the same final number of cell nuclei as control cultures grown in whole embryo extract and dilute horse serum. The protein fraction of the embryo extract could thus be eliminated from the culture fluid without reducing the proliferation rate of strain L mouse cells. It was also demonstrated that neither the ultrafiltrate fraction nor the residue of horse serum could be omitted from the culture medium without diminishing the value of the fluid for proliferation of mouse cells. Of the two serum fractions, the residue in the presence of chick-embryo extract or embryo-extract ultrafiltrate supported a higher rate of proliferation than the serum ultrafiltrate.

In the present study an effort has been made to supplement embryo-extract ultrafiltrate and washed serum residue with known chemicals that might substitute for the ultrafiltrate fraction of the serum. Such a medium has been developed, and the possible role of serum ultrafiltrate in cell proliferation has been defined.

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[1] Received for publication June 10, 1953.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] A more complete bibliography was included in this earlier article. It is regretted that the valuable study of Barski *et al.* (Annales de l'Institut Pasteur 81: 9, 1951) was omitted due to an oversight.

[4] The term "residue" is used in this paper to refer to the material left in the collodion membrane after ultrafiltration.

Materials and Methods

The methods for the preparation and handling of replicate cultures of strain L cells, for measurement of proliferation, for the preparation of components of the medium, for sterilization and storage of solutions, and for chemical analyses have been described (*1*). The serum residue, left in the collodion membrane after ultrafiltration of horse serum, was diluted to original volume with Earle's balanced saline before use. The last lots of serum residue were washed 4 or 5 times during ultrafiltration (see table 1) with volumes of Earle's balanced saline ranging from 250 to 800 ml. per wash to remove more completely the ultrafiltrable components of serum. All materials were sterilized by passage through a Selas filter and were stored in rubber-stoppered test tubes at 3° C.

In all but one of the present experiments the cells were suspended and stirred in embryo-extract ultrafiltrate. Each T-15 flask received 0.5 ml. of the stirred suspension of cells and 2 ml. of the fraction of the culture medium not used in suspending the cells. In the last experiment, since embryo-extract ultrafiltrate was absent from two of the experimental media, the cells were suspended in horse-serum residue.

Synthetic mixture 199 formulated by Morgan, Morton, and Parker (*2*) was prepared as described by the authors. When groups of components were omitted from the mixture, as described later, the osmotic value was adjusted by the addition of the necessary volumes of distilled water or balanced saline of double the concentration of salts.

Results

Four lots of chick-embryo extract, ranging in volume from 595 to 780 ml. each, and five lots of horse serum, ranging from 620 to 1,780 ml. each, were subjected to ultrafiltration (table 1). The yields of embryo-extract ultrafiltrate varied from 92 to 98 percent of the original volume of embryo extract, and the yields of serum ultrafiltrate from 61 to 70

TABLE 1.—*Data on ultrafiltration of embryo extract and horse serum*

Experiment No.:		1	2	3	4, 5	6
Embryo extract	Initial volume (*ml.*)	595	780	655	600	.....
	Period of ultrafiltration (*hours*)	25½	23	22½	21¼	.....
	Percentage of original volume as ultrafiltrate	98	97	92	95	.....
Horse serum	Initial volume (*ml.*)	620	1060	800	750	1780
	Period of ultrafiltration (*hours*)	24½	23	22½*	23¼*	20½*
	Percentage of original volume as ultrafiltrate	70	70	64	66	61

*The period of ultrafiltration was continued for an additional 48 hours at 3° C. during which time the residue was washed 4 or 5 times with volumes of Earle's balanced saline ranging from 250 to 800 ml. per wash.

percent of the original volume of serum used. Samples of all lots of ultrafiltrate were free from protein when tested with sulfosalicylic acid. The nitrogen content of the embryo-extract ultrafiltrate after sterilization ranged from 27 to 34 mg. per 100 ml. (table 2). The nitrogen level of the serum and serum residue after sterilization ranged from 1,044 to 1,129 mg. per 100 ml., and the nonprotein nitrogen of the residue was reduced from approximately 29 mg. per 100 ml. in whole serum to as low as 2.4 mg. per 100 ml. in washed serum residue.

TABLE 2.—*Nitrogen content of horse serum, serum residue, and embryo-extract ultrafiltrate*

Experiment No.	Material					
	Horse serum		Horse serum residue (diluted)		Embryo-extract ultrafiltrate	
	Total nitrogen (*mg. %*)	Nonprotein nitrogen (*mg. %*)	Total nitrogen (*mg. %*)	Nonprotein nitrogen (*mg. %*)	Total nitrogen (*mg. %*)	Nonprotein nitrogen (*mg. %*)
1	1129	29	1080	8	27	27
2	1044	22	1110	9	28	27
3	1070	29	1090	3	28	28
4, 5	.....	.....	1087	2.4	34	33
6	.....	.....	1071	3.2		

In six experiments the lots of embryo-extract ultrafiltrate, serum, and serum residue in combination with mixture 199 and portions thereof were tested on strain L cells. In each experiment the same lots of solution were used on all cultures. The results of the six experiments are summarized in table 3. Each value represents the average of determinations on 4 cultures.

The numbers of nuclei in cultures grown on whole serum, embryo-extract ultrafiltrate, and saline (table 3, column 1) exceeded those of cultures grown on serum residue, embryo-extract ultrafiltrate, and saline (column 2). When, however, mixture 199 was used in place of balanced saline in the latter culture fluid (column 4), the numbers of nuclei in the cultures were significantly increased. When mixture 199 was used with whole serum and embryo-extract ultrafiltrate (column 3), the numbers of nuclei were high, but were consistently slightly lower than when serum residue was used in place of whole serum (column 4). The data were too limited, however, to establish whether this difference was statistically significant.

In an effort to determine what components of mixture 199 supplement the two fractions of serum and embryo extract, selected components of mixture 199 were omitted, and the resultant mixtures were tested on the cells in combination with serum residue and embryo-extract ultra-

TABLE 3.—*Summary of results*

Exp. No.	Inoculum size (No. of cell nuclei in thousands)	Days of exposure to experimental media	Column No.:	1	2	3	4	5	6	7	8	9	10
			Experimental culture media used										
			Horse serum (%)	40		40							
			Serum residue (%)		40		40	40	40	40	40	40	40
			Embryo ultrafiltrate (%)	20	20	20	20	20	20	20	20		
			Saline (%)	40	40							20	60
			Mixture 199 (%)			40	40					40	
			Mixture 199 selected comp. omitted (%)					40 less A.A.*	40 less V.†	40 V.† + A.A.* only	40 A.A.* only		
			Final population (cell nuclei in thousands) after exposure to the experimental culture media defined above										
1	93	4		1,065	1,013	936	1,618	1,069	1,356	1,398			
		10		4,842	2,659	6,697	6,690	2,886	6,931	6,284			
2	154	7		1,564	1,421	2,347	2,934	1,401	3,268	3,038			
		14		6,925	5,360	10,611	10,757	5,725	10,922	11,210			
		21		10,818	9,856	12,268	13,148	9,323	12,358	13,263			
3	144	7		1,537	942	1,662	2,157				1,605		
		14		6,284	1,823	6,947	7,488				7,392		
		21		8,590	5,340	10,027	11,779				10,692		
4	180	7		2,749	1,450		3,726		3,471	3,902	3,290		
		14		8,415	5,825		11,383		10,433	11,444	11,358		
		21		9,294	9,032		9,882		10,538	10,504	9,527		
5	119	7		1,204								1,328	
		14		6,169								3,723	
		21		8,322								4,079	
6	556	5			1,098		2,057					1,468	190
		12			3,903		9,966					4,130	299
		19			7,642		11,307					4,816	133

*A. A. = Amino acids. †V. = Vitamins.

filtrate (table 3, columns 5–8). From the data of the first two experiments, it appeared evident that the amino acids of mixture 199 were the essential supplementing materials. In the third and fourth experiments, the amino acids of mixture 199 were tested alone with washed serum residue and embryo-extract ultrafiltrate (column 8). When the amino acids alone were used as supplements for washed serum residue and embryo-extract ultrafiltrate, the numbers of nuclei in the cultures were still high but slightly lower than when the whole mixture 199 was used as supplement (column 4). For each experiment the difference between the numbers of cell nuclei in cultures grown on the amino acid block as sole supplement, and in cultures grown on the whole mixture 199 as supplement, were not significant; but when both experiments were considered together, the difference was statistically significant. There was a suggestion that the vitamins of mixture 199 served to supplement further the amino acids, but the differences in the numbers of cell nuclei in cultures grown with and without the added vitamins were not statistically significant. For both of these comparisons, an analysis of variance was conducted to provide a basis for statements concerning the significance of differences.

Cells grown in 40 percent serum residue failed to maintain the inoculum number of cell nuclei (column 10). When mixture 199 was added to the serum residue (column 9), cell proliferation occurred but failed to exceed that in cultures grown in dilute serum residue and embryo-extract ultrafiltrate (column 2), and was considerably less than that in cultures grown in serum residue, embryo-extract ultrafiltrate, and mixture 199 (column 4).

Discussion

In this and the preceding study (*1*) an effort was made to determine what components of a serum–embryo extract culture medium are actually used by strain L mouse cells and what components can be eliminated. It was demonstrated that the protein fraction of chick-embryo extract and the ultrafiltrable nonprotein fraction of horse serum can be omitted from the fluid mixture without reducing cell proliferation if the remaining two fractions are supplemented by an amino acid mix. In fact, the resultant mixture of two fractions, embryo-extract ultrafiltrate and diluted serum residue, supplemented with amino acids and possibly with vitamins, supported proliferation of strain L mouse cells better than any culture medium yet tested. It appears that in a serum–embryo extract culture fluid the main role of the serum ultrafiltrate fraction in cell proliferation is to supply amino acids.

Summary and Conclusions

In the present experiments the effects of the protein-containing residue of horse serum have been tested on the proliferation of a strain of mouse cells of single-cell origin. The following results were obtained: Cells failed to maintain the inoculum number of cell nuclei when cultured

in dilute serum residue alone. When either embryo-extract ultrafiltrate or mixture 199 of Morgan, Morton, and Parker was added to the dilute serum residue, cell proliferation occurred to a limited extent. When, however, both of these materials were added to the serum residue, the rate of cell proliferation exceeded that of cells grown in the control fluid of whole serum and embryo-extract ultrafiltrate. Of the components of mixture 199, the amino acid block and possibly to a slight degree the vitamins appeared to be the essential supplementary materials. The results indicate that in a serum–embryo extract culture fluid the main role of the ultrafiltrate fraction of the serum in cell proliferation is to supply amino acids.

It has been demonstrated that the protein fraction of chick-embryo extract and the ultrafiltrable nonprotein fraction of horse serum can be omitted from a serum–embryo extract culture fluid without reducing cell proliferation if the remaining two fractions are supplemented with amino acids. The resultant mixture of embryo-extract ultrafiltrate and supplemented serum residue provides an excellent culture fluid for proliferation of strain L mouse cells.

References

(*1*) Sanford, K. K., Waltz, H. K., Shannon, J. E., Jr., Earle, W. R., and Evans, V. J.: The effects of ultrafiltrates and residues of horse serum and chick-embryo extract on proliferation of cells *in vitro*. J. Nat. Cancer Inst. 13: 121–137, 1952.

(*2*) Morgan, J. F., Morton, H. J., and Parker, R. C.: Nutrition of animal cells in tissue culture I. Initial studies on a synthetic medium. Proc. Soc. Exper. Biol. & Med. 73: 1–8, 1950.

Utilization of Amino Acids, Peptides, and Protein by Cultures of Embryonic Heart [1,2]

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Studies of the nutritional requirements of tissue cultures indicate the importance of both protein components and the dialyzable fraction of the nutrient medium for optimum growth (*1–4*). The relative contributions of protein and nonprotein phases of embryo-extract medium in the growth of chick heart fibroblasts have recently been determined in quantitative balance studies (*5*). The exact nature of the nonprotein nitrogenous substances in embryo extract has not been ascertained, but peptides are present (*6*), in addition to a variety of amino acids.

In the present paper the concentrations of the individual amino acids in embryo extract are reported, as well as the sizes of the total pools of free and conjugated amino acids. Changes in these pools during the cultivation of embryonic chick heart have been determined. In addition a C^{14} balance study has been performed with labeled embryo extract, in order to equate losses of radioactive amino acids, peptides, and protein from the medium with corresponding gains by the explanted tissue. In this connection the rates of incorporation of partial enzymic hydrolysates of labeled protein into tissue cultures were measured.

Materials and Methods

Starch-column chromatography (*7*) was used to determine the amino acids in a trichloroacetic acid extract of whole 11-day chick embryos. After removal of the acid by ether extraction, the solution was made 2 N in HCl, hydrolyzed for 6 hours at 115° C., and the HCl removed by evaporation to dryness *in vacuo*. An aliquot representing 0.05 gm. of fresh embryos was chromatographed, and 0.5 ml. of effluent fractions was collected.

The cultivations were performed as previously described (*5*). Changes in the nonprotein phase of the embryo extract were measured at 2-day intervals. The medium was quantitatively removed from a series of ten culture tubes, and fresh nutrient added to each tube. Picric acid filtrates

[1] Received for publication June 30, 1953.
[2] Aided by a grant from the Iowa Division of the American Cancer Society.

were then prepared from the pooled embryo extract of each time interval, and free amino acids measured according to the Van Slyke ninhydrin-CO_2 method (*8*). Peptides were determined from the increases in the values obtained following hydrolysis.

A labeled embryo extract, prepared (*5*) by injecting glycine-1-C^{14} into fertile eggs (2.6 μc. or 0.2 mg. per egg), was employed in the C^{14} balance study. At 2-day intervals three or four tubes were removed from the series, and the media and tissues pooled separately. The specific radioactivities of the embryo extract and heart proteins (freed of nucleic acids) were determined (*5*), and multiplied by the average mg. of protein per tube to give counts per minute per tube. The C^{14} content of free and total amino acids was measured using the U-tube modification of the Van Slyke method, before and after acid hydrolysis of protein-free filtrates. $C^{14}O_2$ was counted as $BaC^{14}O_3$ (*9*). Conjugated amino acids were calculated by difference.

A highly radioactive protein was prepared by cultivating heart for 6 days in embryo extract containing 8 mg. of added phenylalanine-3-C^{14} (0.5 mc. per mM) per 100 ml. The protein isolated from the heart tissue had 3,200 counts per minute per mg. Assuming a phenylalanine content of 4.0 percent (*10*), the specific activity of the amino acid in this protein was 3,200 $\times$ 25, or 80,000 counts per minute per mg. Portions of the labeled protein were digested for 18 hours at 37° C., with 1/20 part of crystalline pepsin, trypsin, or chymotrypsin at appropriate *p*H. In each case less than 10 percent of the C^{14} was precipitable by trichloroacetic acid. The neutralized digests were sterilized by brief autoclaving, and added at a level of 5 mg. per 100 ml. to embryo extract, for C^{14} uptake experiments. Labeled protein completely hydrolyzed with 6 N HCl was employed as a control.

The C^{14}-amino acids were purchased from Tracerlab, Inc., Boston, on allocation from the U. S. Atomic Energy Commission.

Results and Discussion

Amino acids present in chick-embryo tissues.—Chromatography of the hydrolyzed nonprotein phase of embryo extract (table 1) gave a number of well defined peaks. These accounted for 93 percent of the total ninhydrin color value of the initial solution analyzed. The mg. of total recovered amino acids (including ammonia) divided by 4 gives 94 mg. per 100 ml. of 25-percent embryo extract. No appreciable peaks were observed in the proline, threonine, arginine, lysine, histidine, and cystine regions,[3] while the essential amino acids in the first 3 peaks were present in relatively low concentrations. A very sharp peak at 81 ml. of effluent has been tentatively designated β-alanine, since its rate of emergence was identical with that observed with synthetic β-alanine. This compound was reported also in a dialysate of calf-embryo muscle (*11*).

[3] A relatively small quantity of embryo extract, equivalent to about 200 μgm. of amino acids, was employed in order to give optimum resolution. However, the sensitivity of the colorimetric determinations was lowered, so that any amino acids present in very low concentrations would have escaped detection.

TABLE 1.—*Nonprotein amino acids of 11-day chick embryos*

Ml. of effluent corresponding to peak	Constituent	Concentration in mg. per 100 gm. whole embryos*
14	Leucine-isoleucine	14. 4
16	Phenylalanine	29. 7
23.5	Valine-tyrosine-methionine	22. 6
58	Alanine-glutamic acid	74. 8
72.5	Aspartic acid	70. 5
81	"β-alanine"	28. 5
91.5	Serine	53. 6
95	Glycine	52. 5
103	Ammonia	29. 2
Total		375. 8

*In converting mM to mg., average molecular weights were taken for the 3 unresolved peaks.

The absence of several amino acids in the nonprotein fraction of chick-embryo extract is in accord with the view (*5*) that the protein in the nutrient medium plays an essential role in the growth of tissue cultures.

Utilization of free and conjugated amino acids by heart cultures.—Analysis of a stock 25-percent embryo-extract medium gave a value of 0.220 mg. of COOH-carbon per 2 ml., for amino acids plus peptides. This value was not changed appreciably when the embryo extract was maintained at 37° C. for 2 days in the absence of tissue (table 2). If 110 is taken to be the average molecular weight of the various amino acids in embryo extract (based on the data of table 1), the mg. of total amino acids per 100 ml. of 25-percent embryo extract equals $0.225 \times 50 \times {}^{11}\!/_{2}$, or 103. This last value is in fairly good agreement with that calculated from chromatographic analysis, considering that minor losses occurred in the

TABLE 2.—*Decreases in amino acid and peptide content of embryo extract during cultivation of heart tissue**

Period of cultivation	Total amino acids	Free amino acids	Conjugated amino acids
	mg. COOH-carbon per tube		
Control embryo extract: 2 days without tissue	0. 225	0. 173	0. 052
0–2 days	. 207	. 166	. 041
Decrease	. 018	. 007	. 011
2–4 days	. 187	. 149	. 038
Decrease	. 038	. 024	. 014
4–6 days	. 174	. 133	. 041
Decrease	. 051	. 040	. 011

*The 2 ml. of embryo extract in each culture tube was renewed at 2-day intervals. The losses are calculated in terms of the control values.

latter procedure. The Van Slyke method included both carboxyls of aspartic acid, while the ammonia value has been included in the chromatography calculations.

It may be seen that 23 percent of the nonprotein amino acids in embryo extract (control value in table 2) are in conjugated form. This finding is of interest in relation to the possible role of peptides as intermediates in protein synthesis. It is noteworthy that in protein-free filtrates of adult rat liver, a portion (10 to 20 percent) of each of 9 amino acids studied was in conjugated form (*12*).

Previous work (*5*) has indicated that the nonprotein fraction of embryo extract is the major source of tissue protein during the first 4 days of cultivation, while from the fourth to sixth day the nonprotein and protein phases contribute about equally to the heart protein. The data in table 2 show that both free and conjugated amino acids were taken from the nonprotein phase of embryo extract. The former were utilized to an increasing extent with time of incubation, while the contribution of the peptides stayed relatively constant for the different time intervals.

C^{14} balance with labeled embryo extract.—From a previous study with labeled glycine (*10*) it can be assumed that the radioactivity of the protein and nonprotein amino acids in table 3 represents both glycine-1-C^{14} and serine-1-C^{14}. It can be seen that 40 percent of the labeled amino acids in the original embryo extract were in conjugated form.

During the first 2 days of cultivation the contribution of the labeled nonprotein fraction of embryo extract to the growing tissue slightly exceeded that of the labeled embryo-extract protein. But with increasing time intervals, most of the C^{14} came from the protein phase. These observations agree reasonably well with those reported in an earlier balance study (*5*), which was based on measurement of total amino acids and protein levels.

It is noteworthy that the utilization of C^{14}-peptides of embryo extract exceeded that of free labeled amino acids in each time interval in table 3, and is most marked in the first 2 days. This result differs somewhat from that in table 2, in which the over-all peptide utilization was greater only for the initial phase of cultivation. The C^{14} concentration in heart protein increased progressively with time, while relatively slower changes occurred in the C^{14}-amino acid levels in the fibroblasts. Only traces of conjugated amino acids were detectable in the tissue.

The total losses in C^{14} from embryo extract agreed well with the corresponding gains by the tissue cultures, considering the complexity of the various techniques and also the fact that some of the amino acid molecules may have been metabolized within the fibroblast cells.

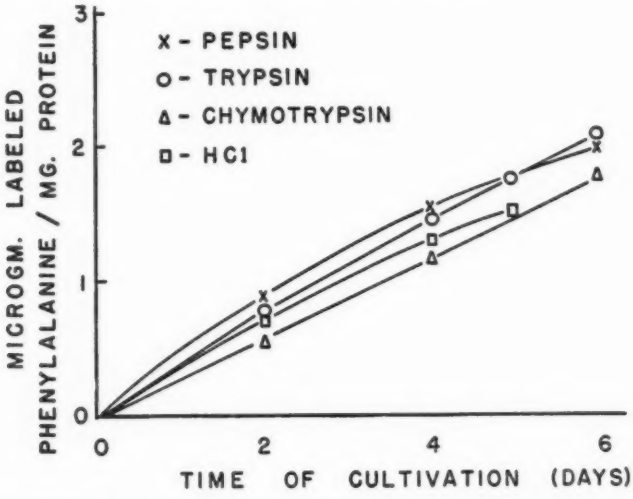
Incorporation experiments with C^{14}-peptides.—When embryo-extract medium was subjected to the action of trypsin, it retained considerable growth-promoting ability. But prolonged cultivation of heart tissue was not possible, inasmuch as the trypsin remaining in the medium attacked the explants. This action could not be counteracted satisfactorily by the addition of crystalline soybean trypsin inhibitor.

TABLE 3.—*Balance of C^{14} following cultivation of heart tissue with labeled embryo extract**

Period of cultivation	Counts per minute per culture tube									
	Embryo extract					Heart tissue				
	Protein	Total amino acids	Free amino acids	Conjugated amino acids	Total loss in C^{14}	Protein	Total amino acids	Free amino acids	Conjugated amino acids	Total gain in C^{14}
Original embryo extracts	2000	326	196	130						
0–2 days	1895	212	174	38		191	24	20	4	
Change	−105	−114	−22	−92	−219	+191	+24	+20	+4	+215
2–4 days	1840	224	156	68		426	37	34	3	
Change	−160	−102	−40	−62	−262	+235	+13	+14	−1	+248
4–6 days	1785	209	144	65		736	45	39	6	
Change	−215	−117	−52	−65	−332	+310	+8	+5	+3	+318

*For each time interval changes in embryo extract were calculated in terms of the original medium, since the latter was renewed every 2 days. The C^{14} content of the heart tissue was cumulative, and hence comparisons were made of consecutive values.

Another approach was the addition to stock embryo extract of labeled peptides, prepared from the partial proteolytic digestion of radioactive protein. The latter was obtained from heart tissue that had been cultivated in the presence of phenylalanine-3-C^{14}. Text-figure 1 shows that the phenylalanine-C^{14} of partially hydrolyzed protein was rapidly incorporated into the protein of heart cultures. The 6-day values represent uptakes of approximately one-third of the total C^{14} employed in the 3 changes of medium. It may be seen that the rates are not essentially different from that with acid-hydrolyzed labeled protein.



TEXT-FIGURE 1.—Utilization of free and peptide-bound labeled phenylalanine by cultures of embryonic heart. Each point represents 2 or 3 tubes.

Since the 3 proteinases split rather different types of linkages, it is likely that the phenylalanine peptides differed considerably in their composition in the 3 cases. The fact that the rates of C^{14} incorporation are not markedly different and do not differ significantly from that with free phenylalanine suggests that the peptides may have been hydrolyzed prior to utilization in tissue protein synthesis. However, the data do not rule out the possibility that the cultures incorporated peptides directly into protein structures. Several types of experiments point to a utilization of embryo-extract protein by heart fibroblasts without complete prior hydrolysis. Further work is needed to clarify this important question.

Summary

Twenty-five percent chick-embryo extract, used as the nutrient medium for the cultivation of chick heart fibroblasts, contained approximately 100 mg. of nonprotein amino acids per 100 ml. Almost one-fourth of these were in conjugated form. During cultivation both free and conjugated amino acids, as well as protein, were removed from the medium. When

labeled embryo extract containing C^{14}-amino acids, peptides, and protein was employed, it was possible to equate C^{14} losses from these fractions with corresponding gains by the heart cultures. Partial proteolytic digests and complete acid hydrolysates of C^{14}-labeled protein were rapidly incorporated into tissue cultures, and at approximately equal rates, when added to the nutrient medium.

References

(*1*) JENSEN, A. B., and HARRIS, L.: The behavior of chick heart fibroblasts in some protein-free media. Exper. Cell Res. 3: 706–709, 1952.

(*2*) FISCHER, A.: Amino-acid metabolism of tissue cells *in vitro*. Biochem. J. 43: 491–497, 1948.

(*3*) GERARDE, H. W., and JONES, M.: The effect of cortisone on collagen synthesis in vitro. J. Biol. Chem. 201: 553–560, 1953.

(*4*) BARSKI, G., MAURIN, J., WIELGOSZ, G., and LEPINE, P.: Conditions de nutrition cellulaire *in vitro* en culture sans support plasmatique. Rôle des fractions micro- et macromoléculaires. Ann. Inst. Pasteur 81: 9–32, 1951.

(*5*) FRANCIS, M. D., and WINNICK, T.: Studies on the pathway of protein synthesis in tissue culture. J. Biol. Chem. 202: 273–289, 1953.

(*6*) WESTFALL, B. B., PEPPERS, E. W., and EARLE, W. R.: A chromatographic investigation of the peptides and amino acid content of the protein-free ultrafiltrate from horse serum and chick embryo extract. Paper presented at the March 1952 meeting of the American Tissue Culture Association, Providence, Rhode Island.

(*7*) MOORE, S., and STEIN, W. H.: Chromatography of amino acids on starch columns. Solvent mixtures for the fractionation of protein hydrolysates. J. Biol. Chem. 178: 53–77, 1949.

(*8*) HAMILTON, P. B., and VAN SLYKE, D. D.: The gasometric determination of free amino acids in blood filtrates by the ninhydrin-carbon dioxide method. J. Biol. Chem. 150: 231–250, 1943.

(*9*) WINNICK, T.: Studies on the mechanism of protein synthesis in embryonic and tumor tissues. Arch. Biochem. 27: 65–74, 1950.

(*10*) GERARDE, H. W., JONES, M., and WINNICK, T.: Protein synthesis and amino acid turnover in tissue culture. J. Biol. Chem. 196: 51–68, 1952.

(*11*) GORDON, A. H.: An investigation of the intracellular fluid of calf embryo muscle. Biochem. J. 45: 99–105, 1949.

(*12*) SOLOMON, J. D., JOHNSON, C. A., SHEFFNER, A. L., and BERGEIM, O.: The determination of free and total amino acids in rat tissues. J. Biol. Chem. 189: 629–635, 1951.

Effects of Ionizing Irradiation Treatments on Tumor Regression [1]

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The pronounced regression of a transplanted lymphosarcoma of the mouse caused by synergistic action of simultaneous body and tumor X irradiation was described previously (*1*). In a subsequent report (*2*) data on the nature of the systemic effect, *i.e.* the role played by the irradiated body in tumor regression, and methods of influencing tumor regression following local tumor irradiation were presented. In the present communication, dose-response curves for the regression of the transplanted lymphosarcoma are given for various experimental conditions. In addition, the dose-response curves for a transplanted mammary carcinoma in C3Hb mice were studied. They show that the response pattern is not specific for lymphosarcoma.

Experimental Methods

One series of experiments was conducted with strain A male mice between three and four months of age bearing a transplanted lymphosarcoma described previously (*1*, *2*). The 91st to 100th generation transplants were used. Ten days following transplantation the animals were X irradiated with a 186 KVP, 20 ma. beam filtered with 0.25 mm. copper plus 0.55 mm. aluminum at a distance of 25 cm. The dose rate under these conditions was 226 r per minute in air. The tumor volume was determined by caliper measurement.

In the first experiment, Ia, tumor regression was studied when the tumor dose was held constant while the body dose was varied. The tumor and body were irradiated simultaneously. The animals were confined in lucite holders, the tumors exteriorized and irradiated with 1,000 r. Various thicknesses of copper and lead shields covered the holders to vary the body dose. The body dose of the various groups is seen in table 1.

In the second experiment, Ib, tumor response to local irradiation was studied. Four groups of mice were placed in lucite holders, and the tumors and skin flaps were drawn outside of the holder. The bodies of

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

TABLE 1.—*Filtration used to obtain various body doses for a given tumor dose in total-body irradiation*

Ia subgroup	Body shield		Dose to body
	Cu (*mm.*)	Pb (*mm.*)	(*r*)
1	0	5.0	0
2	2.0	0.55	49
3	0.25	0.55	103
4	2.0	0	229
5	1.0	0	387

the mice were shielded with 5 mm. lead and the tumors were exposed to a single dose of 400, 1,000, 2,000, 4,000 or 6,000 r.

The third experiment, Ic, consisted of keeping the body dose constant at 100 r while the dose to the tumor was varied from 400 to 2,000 r. This was done by varying the thicknesses of the shields covering the holders, as shown in table 2.

TABLE 2.—*Filtration used to obtain various tumor doses for a given body dose in total-body irradiation*

Ic subgroup	Body shield		Dose to tumor
	Cu (*mm.*)	Pb (*mm.*)	(*r*)
1	2.0	0	400
2	0.50	0.47	720
3	0.25	0.55	912
4	2.00	0.55	2,000

In the fourth experiment, Id, tumor regression following total-body irradiation, *i.e.* irradiation of the whole animal including the tumor, was re-examined where tumor and body were irradiated simultaneously (*1*). In one series the animals in the lucite holders were irradiated with 100, 200, 300 or 400 r. In another series, the animals, again in the holders, were irradiated with 100, 250, 400, 600 or 800 r, and were given homologous bone marrow (*3*) immediately following irradiation. In the third series the mice again received 100, 250, 400, 600 or 800 r, but their spleens were surgically exteriorized and shielded with lead during irradiation, using the method described by Jacobson (*4*).

A similar set of experiments was done with C3Hb male mice three to four months of age bearing a transplanted, poorly differentiated adenocarcinoma of the mammary gland, designated C3HBA. This tumor was obtained from Dr. M. K. Barrett in its 120th generation (*5*) and transplants from seven subsequent generations were used. The animals were treated approximately 15 days after subcutaneous transplantation. The

basic irradiation conditions were the same as those in the lymphosarcoma experiments.

This tumor is not as radiosensitive as the lymphosarcoma. In the first series of irradiations, IIa, the tumor dose was held constant at 2,000 r while the body dose was varied from 0 to 416 r. The necessary shields for the bodies are shown in table 3.

TABLE 3.—*Filtration used to obtain various body doses with the tumor dose constant in total-body irradiation*

IIa subgroup	Body shield		Body dose
	Cu (*mm.*)	Pb (*mm.*)	(*r*)
1	0	5	0
2	1. 75	1. 10	52
3	0. 75	0. 81	106
4	1. 00	0. 34	276
5	2. 50	0	416

In the second experiment, IIb, the tumors were treated with 2,000, 4,000 or 6,000 r. The mice were confined in the lucite holders and covered with the 5-mm. lead shields as described previously.

In the third experiment, IIc, the body dose was held at 100 r while the tumor dose was varied from 845 to 6,100 r. Again, the shields of varying thicknesses of copper and lead were placed over the lucite holders to keep the body dose at 100 r, as seen in table 4.

TABLE 4.—*Filtration used to obtain various tumor doses with the body dose constant in total-body irradiation*

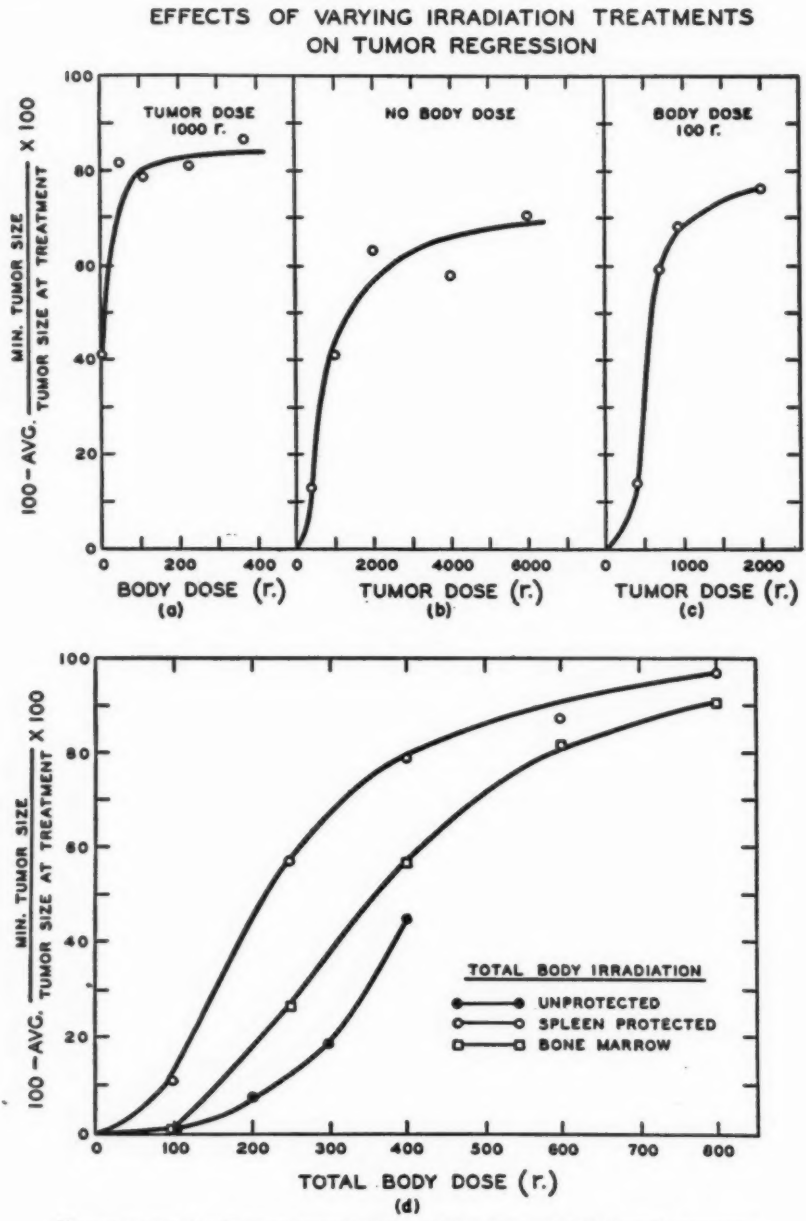
IIc subgroup	Body shield		Tumor dose
	Cu (*mm.*)	Pb (*mm.*)	(*r*)
1	0. 50	0. 55	845
2	0. 75	0. 81	2, 149
3	0. 75	1. 10	2, 910
4	1. 00	1. 57	4, 588
5	1. 50	1. 57	6, 102

In the last experiment, IId, the whole animal was irradiated with 200, 400, 600 or 800 r with the spleen shielded.

Results

The average ratio of the minimum size to which the tumor regressed to the size of the tumor at treatment was used as a basis for comparison.

In most experiments the larger the dose the greater was the tumor regression, up to a certain dose beyond which no appreciable increase in



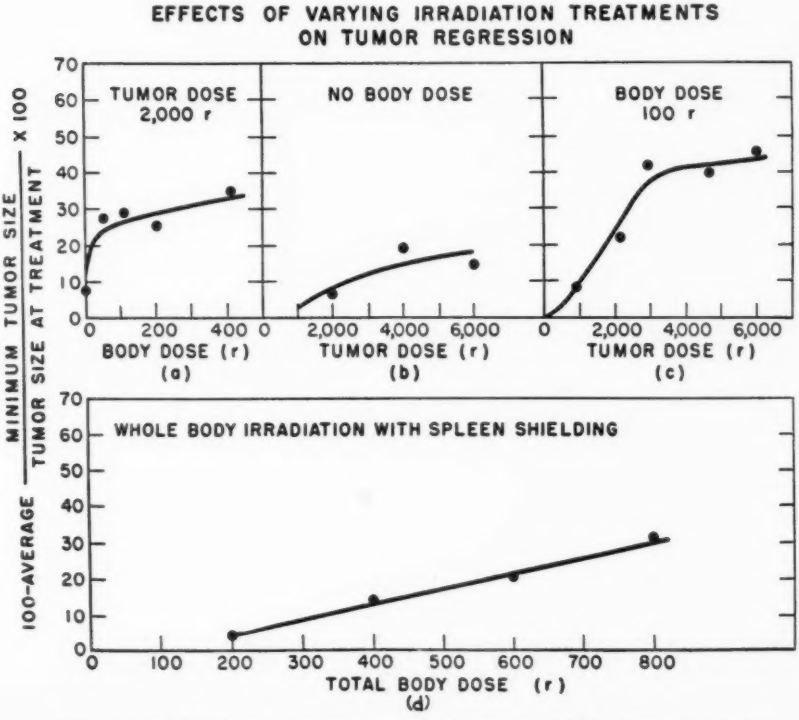
TEXT-FIGURE 1.—Dose-response curves of the transplanted lymphosarcoma.

tumor regression was seen (text-figs. 1 and 2, tables 5 and 6). The animals receiving 1,000 r to the tumor and no body irradiation in the Ia and Ib experiments showed similar response and the combined data of

these two experiments are presented in table 5 and text-figure 1. One exception to the leveling-off process occurred in the C3HBA tumor response to total-body radiation. Apparently, a maximum response was not reached at 800 r, while the response curve to total-body irradiation of the lymphosarcoma leveled off after 600 r. The spleen-shielded animals showed greater tumor regression than did the bone-marrow-treated animals or the nonprotected animals.

The increase in response (text-fig. 1d) following spleen shielding occurred, apparently, because of the surgical operation. A group of sham spleen-protected animals was irradiated with 400 r together with spleen-protected animals and nonoperated animals. The tumors of the sham spleen-protected group regressed 90 ± 2.6 percent of treatment size, those of the spleen-protected animals 80 ± 6.1 percent, and those of the nonoperated animals 64 ± 4.9 percent.

When the tumor dose was held constant and the body dose was varied, a plateau in the lymphosarcoma regression curve occurred with body doses greater than 50 r. When the lymphosarcoma was irradiated alone, the regression curve started to level off at a tumor dose of 2,000 r; when the tumor was irradiated with 100 r to the body the curve started to level off at a tumor dose of about 1,000 r.



TEXT-FIGURE 2.—Dose-response curves of the transplanted adenocarcinoma.

TABLE 5.—*Effects of varying irradiation treatments on lymphosarcoma regression*

Experimental group	X-ray dose: tumor (r)	X-ray dose: body (r)	Number of animals	Average ratio = R $\left(\frac{\text{Tumor minimum}}{\text{Tumor at treatment}} \times 100\right)$	100 − R	Average tumor size at treatment
Ia	1,000	0	19	59.4 ± 6.5*	40.6	1,190 ± 550†
	1,000	49	10	18.4 ± 5.1	81.6	1,600 ± 590
	1,000	103	19	21.4 ± 4.7	78.6	1,860 ± 870
	1,000	229	10	18.6 ± 5.4	81.4	1,760 ± 700
	1,000	387	20	13.4 ± 3.1	86.6	1,670 ± 630
Ib	400	0	10	86.5 ± 4.1	13.5	2,561 ± 600
	1,000	0	19	59.4 ± 6.5	40.6	1,190 ± 550
	2,000	0	20	37.2 ± 5.7	62.8	3,180 ± 1,230
	4,000	0	20	41.9 ± 7.1	58.1	1,390 ± 220
	6,000	0	10	29.0 ± 8.2	71.0	3,840 ± 1,220
Ic	400	100	20	85.6 ± 3.8	14.4	1,260 ± 570
	720	100	10	40.5 ± 8.1	59.5	1,520 ± 400
	912	100	7	31.7 ± 7.5	68.3	1,250 ± 530
	2,000	100	15	23.5 ± 6.2	76.5	1,290 ± 590
Id_1 Bone marrow	100	100	10	99.5 ± 0.5	0.5	1,500 ± 630
	250	250	10	72.5 ± 4.6	27.5	2,100 ± 640
	400	400	10	43.5 ± 4.3	56.5	1,710 ± 610
	600	600	22	18.0 ± 1.9	82.0	1,700 ± 290
	800	800	13	8.7 ± 1.9	91.3	890 ± 410
Id_2 Spleen protected	100	100	8	89.0 ± 4.1	11.0	1,070 ± 500
	250	250	8	42.7 ± 2.1	57.3	990 ± 130
	400	400	26	20.7 ± 3.1	79.3	930 ± 500
	600	600	19	12.7 ± 3.6	87.3	1,250 ± 560
	800	800	16	3.4 ± 1.3	96.6	1,150 ± 570
Id_3	100	100	10	99.4 ± 0.6	0.6	2,250 ± 770
	200	200	10	92.3 ± 2.0	7.7	1,870 ± 730
	300	300	10	81.1 ± 4.1	18.9	2,160 ± 410
	400	400	18	55.4 ± 2.2	44.6	1,680 ± 980

*Standard error.

†Standard deviation.

TABLE 6.—*Effects of varying irradiation treatments on adenocarcinoma regression*

Experimental group	X-ray dose: body (r)	X-ray dose: tumor (r)	Number of animals	Average ratio = R $\left(\frac{\text{Tumor minimum}}{\text{Tumor at treatment}} \times 100\right)$	100 − R	Average tumor size at treatment
IIa	0	2,000	8	91.0 ± 5.7*	9.0	700 ± 240†
	52	2,000	17	70.5 ± 4.3	29.5	980 ± 500
	106	2,000	15	70.6 ± 6.3	29.4	820 ± 400
	276	2,000	17	73.8 ± 5.5	26.2	900 ± 600
	416	2,000	16	64.7 ± 5.3	35.3	810 ± 400
IIb	0	2,000	10	93.4 ± 3.6	6.6	1,260 ± 700
	0	4,000	8	82.0 ± 5.9	18.0	600 ± 300
	0	6,000	10	85.0 ± 5.8	15.0	840 ± 420
IIc	100	845	17	90.7 ± 3.5	9.3	1,120 ± 640
	100	2,149	18	77.9 ± 5.0	22.1	980 ± 680
	100	2,910	17	57.6 ± 3.9	42.4	1,040 ± 100
	100	4,588	9	58.3 ± 7.8	41.7	910 ± 400
	100	6,102	13	54.9 ± 7.0	45.1	730 ± 400
IId	200	200	19	97.0 ± 1.8	3.0	1,170 ± 790
	400	400	18	87.8 ± 3.9	12.2	1,620 ± 950
	600	600	18	80.3 ± 3.9	19.7	1,600 ± 1,000
	800	800	17	68.5 ± 4.7	31.5	1,200 ± 540

*Standard error.

†Standard deviation.

Although the C3HBA tumor is less radiosensitive than the lymphosarcoma, a systemic effect of irradiation plays a part in this tumor regression also. When the tumor was irradiated locally with 2,000 r and the body received no irradiation, the tumor regressed 9 percent of its treatment size. On the other hand, when the tumor received 2,000 r and the body 50 r, it regressed, on an average, 30 percent of treatment size. As in the case of the lymphosarcoma, 50 r was sufficient to produce a maximum systemic effect. Whether the C3HBA tumor was irradiated alone or with an additional dose to the body, the curves level off after 3,000 r to the tumor (text-fig. 2b and 2c).

Discussion

This series of experiments has resulted in typical dose-response curves. The leveling off with increased dose, however, is not seen in the total-body radiation dose-response of the C3HBA tumor (text-fig. 2d). This tumor does not respond even to local irradiation below 1,000 r and whole-body irradiation is limited by its lethal effect to less than 1,000 r. Similarly the curve of total-body dose versus lymphosarcoma response does not level off abruptly because here again the maximum tumor response has not been reached (text-fig. 1d). It is interesting to note that the total-body irradiation of 800 r is more effective than 6,000 r to the lymphosarcoma alone. When animals received up to 400 r to the body, along with a high tumor dose (1,000 r), response was not as great as with 800 r total-body irradiation.

Although the size of the lymphosarcomas in the animals receiving whole-body irradiation is, on the whole, smaller than the size of the lymphosarcomas in the other groups, the great response here cannot be laid to the small size of these tumors. The tumors which at treatment are below the average size do not, in the majority of cases, regress more on a percentage basis than the tumors above the average size. This is also true of the mammary tumor response. Furthermore, it has previously been shown that regression is not dependent on size of the tumor at treatment (*1*) in the range used.

Perhaps the most remarkable point found in this series is the fact that 50 r total-body irradiation, in addition to local tumor irradiation, is sufficient to produce a maximum systemic effect for both tumors. A response to such a low body dose suggests an adrenal-cortical stimulation. The increased regression produced by the mobilization of the spleen also points toward an adrenal-cortical stimulation produced by an operative trauma. However, preliminary data using adrenalectomized tumor-bearing animals indicate that tumors in adrenalectomized and sham-adrenalectomized animals respond in the same way to whole-body irradiation.

Reducing the body dose by placing the lead and copper shields over the mice changes the quality of the radiation the body receives. The change in wave length is not great enough, however, to change the biologic effects.

Any soft radiation arising from the lead or copper is removed by the lucite, so the animals still get a uniform body dose.

The results obtained with these two tumors originating from different tissues, and the similar results obtained in additional studies of another mammary tumor originating in strain A mice, show that the systemic effect is not dependent on the lymphoid origin of the tumor that was studied previously (*1*).

Summary

Dose-response curves were obtained for a localized lymphosarcoma and a mammary carcinoma for varying irradiation conditions. These consisted in varying *a*) total-body irradiation, *b*) local tumor irradiation, *c*) local tumor irradiation with the dose to the body held constant, and *d*) body irradiation with the dose to the tumor held constant.

It was found that with increasing dose the response of the tumor, expressed as a decrease in tumor volume, increased initially and then leveled off with increasing dose for all irradiation conditions employed except in the following instance. In total-body irradiation of the C3Hb mice bearing the mammary carcinoma a maximum response had not been reached at 800 r. However, the lymphosarcoma response was just about maximum at 800 r total-body irradiation. In local tumor irradiation 2,000 r was sufficient to produce a maximum effect in the lymphosarcoma and 4,000 r was sufficient to produce a maximum response in the mammary carcinoma.

When the body dose was constant while the tumor dose was increased, the curve leveled off between 1,000 r and 2,000 r in the case of the lymphosarcoma, and between 3,000 r and 4,000 r in the case of the mammary carcinoma.

When the tumor received a constant dose while the dose to the body was increased, 50 r was found sufficient to produce a maximum effect for both tumors.

Bibliography

(*1*) Hollcroft, J., Lorenz, E., and Hunstiger, H.: Effects of ionizing radiations on a transplanted lymphosarcoma. J. Nat. Cancer Inst. 11: 1–16, 1950.

(*2*) Hollcroft, J. W., Lorenz, E., and Matthews, M.: Factors modifying the effect of X-irradiation on regression of a transplanted lymphosarcoma. J. Nat. Cancer Inst. 12: 751–763, 1952.

(*3*) Lorenz, E., Congdon, C., and Uphoff, D.: Modification of acute irradiation injury in mice and guinea-pigs by bone marrow injections. Radiology 58: 863–877, 1952.

(*4*) Jacobson, L. O., Simmons, E. L., Marks, E. K., Robson, M. J., Bethard, W. F., and Gaston, E. O.: The role of the spleen in radiation injury and recovery. J. Lab. & Clin. Med. 35: 746–770, 1950.

(*5*) Barrett, M. K., and Morgan, W. C.: A maternal influence on the growth rate of a transplantable tumor in hybrid mice. J. Nat. Cancer Inst. 10: 81–88, 1949.

Mobile Resistance Factor in Acquired Immunity to Homologous Tissue Transplantation [1]

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The purpose of this paper is to offer experimental data giving support to the hypothesis of the existence of a mobile resistance factor in acquired immunity to homologous [3] tissue transplantation.

In previous work from this laboratory (*1*, *2*), the lack of an intrinsic cellular incompatibility on the one hand and the existence of a mobile resistance factor on the other were indicated in primary immunity. The present paper is an extension of the previous work and presents data of a similar nature regarding secondary or acquired homologous tissue immunity.

Methods and Materials

In essence, the procedure consisted of immunizing half of a group of inbred mice to a particular transplantable tumor. Approximately one month after immunization, skin grafts were exchanged between immunized and nonimmunized animals. Four to five weeks following skin grafting, the grafts in both sets of mice were challenged (*i.e.*, inoculated intradermally) with a fragment from a subsequent transplant generation of the same tumor. This experimental plan is shown diagrammatically in text-figure 1. Appropriate control series were also established as is shown in the table of results (table 1).

From a study of the plan, it can be seen that if the tumors were found to grow within the skins grafted from nonimmune mice to immune mice, we would have to conclude that mobile resistance factors were not present; if, on the other hand, the tumors failed to grow there, the possible presence within the graft of resistance factors from the recipient animal would be demonstrated. Likewise, and in similar fashion, the presence or absence

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[1] Received for publication June 12, 1953.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Definitions of terms:

Autologous—referring to one and the same animal.

Isologous—referring to animals of identical or almost identical genotype (*i.e.* identical twins or inbred animals of the same or *very* closely related strains).

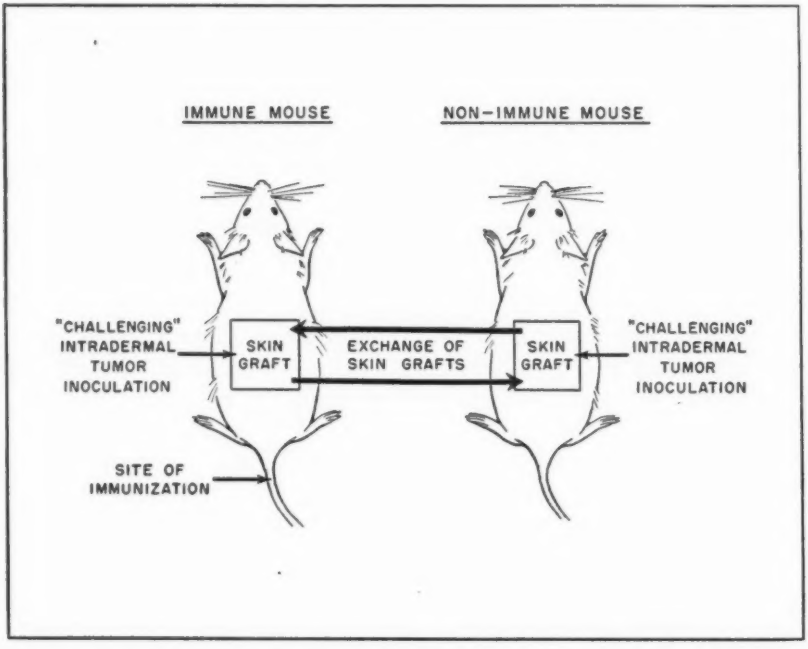
Homologous—referring to animals of one species but of different genotypes.

Heterologous—referring to animals of different species.

TABLE 1.—*Results of three experiments**

Exp. No.	Tumor and mouse strain	Immune mice				Nonimmune mice			
		Immune skin grafts		Nonimmune skin grafts		Immune skin grafts		Nonimmune skin grafts	
		Number of takes	Number of mice	Number of takes	Number of mice	Number of takes	Number of mice	Number of takes	Number of mice
1	Sarcoma 49..... BALB/c.....	3	23	4	17	15	15	18	18
2	Sarcoma 37..... BALB/c.....	9	20	2	19	16	19	20	22
3	Sarcoma 37..... C3H.....	3	15	5	8	10	12	12	15
	Total.....	15	58	11	44	41	46	50	55
	Percent takes....	26		25		89		91	

*Results were recorded at the following time intervals after intragraft tumor inoculation: Experiment 1—2 weeks, Experiment 2—3 weeks, Experiment 3—12 days.



TEXT-FIGURE 1.—Diagrammatic scheme of the experimental procedure.

of local or fixed resistance factors in the transplanted skins would be indicated.

In each separate experiment of this type, male mice 3 to 6 months of age and of one particular inbred strain were utilized. The strains are indicated in table 1. The tumors used, as is also indicated in the table, were DBA sarcoma 49 (*3*) and Sarcoma 37.

The immunization procedure was essentially that described by Andervont (*4*) and consisted of the intracaudal injection of approximately 0.005 cc. of tumor mince. In the first two experiments, the inoculated tails were amputated 10 to 14 days after the inoculation, whether or not tumor growth had occurred. (Tumor growth occurred in over 90 percent of these animals.) In the third experiment, in which Sarcoma 37 was inoculated into strain C3H mice, very few animals showed even temporary gross tumor growth in their tails; consequently, the tails of the mice in the third experiment were not amputated.

The technical details of the skin grafting have been discussed in a previous publication (*5*), and were quite similar to those described by Randall (*6*). A detailed description of the intradermal tumor inoculation technique has also been published (*1*). In each experiment, successive inoculations were alternated among the several groups, in order to ensure comparable inocula in each.

A random sample of those animals exhibiting tumor growth within the skin grafts was used for microscopic study of the implant *in situ*. In each case the tumor implant was entirely within the skin graft, as was demonstrated by the intact transplanted panniculus carnosus muscle. From the gross appearance of these tumors it is possible to state with considerable certainty that this condition existed in all or almost all cases of tumor growth, at least until the time at which the data were recorded. However this condition, *i.e.* the complete containment of the tumor implant within the grafted skin, was not essential to the conclusions that were drawn, as will be pointed out in the discussion.

Results and Discussion

A "take," for the purposes of this paper, is defined as a grossly visible tumor nodule present at the time of recording. In the case of the DBA sarcoma, almost all intradermal tumors ultimately regressed. Sarcoma 37 exhibited progressive, *i.e.* continuous, growth in most of the animals of strain BALB/c in which "takes" were recorded; but, because of the intradermal site of inoculation, it showed ultimate regression in all cases in the C3H strain. The data in each experiment were recorded at a time interval after tumor implantation that showed the greatest variation in results among the different series. The actual intervals are shown in table 1.

The results of the experiments can be easily perceived from the data presented in table 1. The tumor growths failed to show the effects of any intragraft immunity in all series of nonimmunized mice, regardless of the source of the skin grafts. On the other hand, they did show the

effects of intragraft immunity in all series of immunized mice, again regardless of the source of the skin grafts. The number of animals used was sufficient to make these results statistically highly significant, and the fact that three genetically different systems were used means that the data can, with confidence, be used as a basis for generalizations.

It will be noted that these results are similar to those obtained in the previous studies on primary immunity (*1*). The generalization that a specific mobile resistance factor *does* exist in homologous tissue transplantation in the mouse is supported by these results and those of the previously reported experiments of this series (*1*, *2*). No sessile resistance factor could be detected by the use of the methods employed. Possible objections to these conclusions are discussed in a subsequent paragraph.

It must be emphasized that the data presented do not characterize the resistance factor or factors in any way other than as to mobility. Even the mobility is not well defined for the data do not answer precisely the question, "How mobile?" Resistance factors sufficiently mobile to invade, within a period of 1 month, the skin grafts taken from nonimmune animals were conclusively demonstrated. Further work will be required to determine more precisely the degree of mobility.

Although it is doubtless true that stromal elements from the hosts invaded the skin grafts, the data argue that the resistance acquired by the skins taken from nonimmunized mice and grown in immunized hosts was not intimately associated with such an invading stroma (*i.e.* was not attached to the invading stroma in a sessile sense). Had immune factors invaded the grafts along with, and intimately associated with, the stromal elements, rather than by some more freely mobile method, the skins transplanted from immunized to nonimmunized mice would presumably have shown some sign of immunity. Of course, the transplantation procedure might have destroyed resistance factors associated with stromal elements in the grafted skins. This argument cannot be answered with absolute assurance. However, it can be pointed out that most stromal elements, with the exception of nerve axons, do survive in skin grafts. Thus, had there been immune factors intimately associated with the invading stroma in a sessile sense, it seems probable that they would have survived transplantation and would have been observable in the skins from immune animals transplanted to nonimmune animals. On the other hand, the possible role of invading nerve fibers in these experiments demands further consideration in view of recent claims that nerves play a significant part in immune phenomena (*7*, *8*).

It should be pointed out that despite good evidence that the intradermal tumor inoculations were actually made into the grafts rather than the hosts, it is possible that some tumor cells may have escaped from the confines of the skin grafts. It must be considered certain, however, that most, if not all, were actually deposited within the grafts. The assumption that a small percentage of tumor cells in each case escaped from the grafts would not alter the conclusions to be drawn from the data. Thus, had there not actually been a mobile resistance factor, tumor cells implanted

in skins taken from nonimmunized mice and grafted to immunized mice would have grown, regardless of the escape of some cells into the tissues of the immune hosts. Likewise, a fixed resistance factor, had it existed, would have produced some degree of inhibition of tumor growth when tumor was inoculated into the skins taken from immunized mice and grafted to nonimmune, even if a few cells had escaped from the graft. It has been shown by a number of workers (*9*, *10*) that in the transplantation of immunizing tumors such as those used in the present study, the growth of the tumor is markedly dependent upon dosage. Thus, in order to mask completely the effects of an immunity associated with the grafted skin, most if not all of the tumor inoculum would have had to be outside the graft. This assuredly did not happen.

Previous reports in the literature have been contradictory. Despite reports by Gorer (*11*) that mice can produce a variety of different types of agglutinins in response to immunization with homologous tumor tissue, and despite the existence of acquired blood-cell antibodies such as those to the Rh factor in the human, there have been few successes in homologous systems in demonstrating circulating resistance factors to cell types other than the red cell.

Successful demonstration of circulating cytotoxins in immunity to homologous leukemia was reported by Gorer (*12*), who incubated the leukemic cells with homologous antisera prior to implantation into susceptible hosts. Similarly, Kidd (*13*, *14*) has reported the suppression of growth of the Brown-Pearce rabbit tumor by incubation with homologous antisera. Burmester (*15*) has shown a cytotoxic effect of avian tumor homologous antiserum. Lumsden (*16*), while trying to prove the existence of cancer-specific antibodies, showed the presence in tissue culture of homologous cytotoxins. Similar work by Phelps (*17*) demonstrated rat anti-rat-tumor cytotoxins in tissue culture. Takeda (*18*) and other Japanese authors have recently induced specific resistance to the Yoshida sarcoma in homologous serum. These 6 sources are the only ones the authors have been able to find that have claimed the *direct* demonstration of cytotoxins in completely homologous systems. It could possibly be argued that in the cases of Burmester, Kidd, and Gorer and in the work with Yoshida sarcoma the antibody might have been specifically directed against an extrinsic virus rather than directly against the tissue cells.

On the other hand, there have been many reported and probably many unreported failures. Lambert and Hanes (*19*) showed that rat sarcoma grew as well in immune rat plasma as in normal plasma. Gussio (*20*), Mottram and Russ (*21*), and Leitch (*22*) failed to inhibit rat tumors by incubation with immune rat sera. Weil (*23*) was unable to demonstrate antibody by obtaining anaphylaxis in guinea pigs inoculated with rat anti-rat-sarcoma sera and challenged with the sarcoma. Harris (*24*), as the result of tissue culture experiments, concluded that "cytotoxins, while active against heterografts, do not appear in homoplastic transplantation." Lewis *et al.* (*25*) could find no evidence of homologous cytotoxins by the incubation technique. Medawar (*26*), on the basis of tissue culture ex-

periments, concluded: "Notwithstanding statements that have from time to time been made to the contrary, the results justify the conclusion that the occurrence of free serum antibodies is not a sufficient explanation of the destruction of foreign homologous tissue." As the result of recent work Allgower *et al.* (*27*) reached a similar conclusion.

It is apparently quite important in this type of work to distinguish carefully between experiments in homologous and in heterologous systems. Although there have been reports of failure, it is apparently a much simpler task to discover circulating cytotoxins in heterologous transplantation, and such reports are numerous.

Indirect methods such as parabiosis for the detection of circulating homologous resistance factors have not been much more successful than the direct methods. In the field of tissue transplantation, parabiosis has, in general, raised more questions than it has answered. The technique has been used extensively in the investigation of a variety of physiological problems (*28*) without much regard to the genotypic relationships of the paired animals. Why such parabioses are often successful, when free grafts between the animals would not be, is an important but almost completely uninvestigated phenomenon.

Experiments utilizing parabiosis to study the nature of homologous transplantation immunity have not been numerous in modern times. Kross (*29*) united susceptible with naturally immune rats but was unable to demonstrate the transfer of any protective agent. Furth *et al.* (*30*) showed that the growth of leukemia was not influenced by union of the susceptible with a resistant mouse despite the proved existence of vascular anastomoses between the animals. He concluded that "it is unlikely that humoral factors are involved." Cloudman (*31*) obtained unique results which may have been dependent on the particular genetic relationships he employed. He concluded that: "1) Parabiosis between different stocks of mice is possible. 2) Parabiosis changes to susceptibility the normal resistance of black mice to transplants of tumor C198! 3) Changed reaction of a previously nonsusceptible mouse following parabiosis with a susceptible mouse indicates a response like that in an F_1 hybrid and the result is called pseudo-hybridization." Harris (*32*), using parabiosis between mice of the same strain, found that both animals became immune if an immunizing tumor were inoculated into one. However, he was of the opinion that it was an antigen rather than an antibody that passed between the animals.

The most extensive work on homologous parabiosis in transplantation immunity has been reported by Bichel and Holm-Jensen (*33*). These authors showed that in *isologous* parabiosis of a mouse immunized against a transplantable homologous leukemia, with a mouse unimmunized, the unimmunized mouse was uniformly resistant to challenge with the leukemia. There can be little doubt that resistance was transferred in these experiments. However, it seems that there are two possible objections to the conclusion that the results were necessarily due to the transfer of antitissue antibodies. The first is the same one registered in the case of

the tissue culture and incubation experiments cited previously, that leukemic leukocytes are suspected of harboring associated viruses (which may or may not be related to the etiology of the disease). Contaminating viruses have been reported in leukoses by Taylor and MacDowell (*34*) and by Law (*35*). If such a virus had been contained in the leukemic leukocytes used by Bichel and Holm-Jensen, the "antibodies" that were transferred between the parabionts could well have been antivirus rather than antitissue bodies. The second possible objection is inherent in the method of parabiosis and involves the question of whether the resistance that was transferred from one parabiont to the other was of an active or passive nature. Although the exact time intervals between immunization of one parabiont and the challenging of the other were not always reported, it was probably a matter of from a few days to three weeks. Thus, circulating "antigen" could have caused an active immunization of both animals, especially if a cell fragment or particulate could be antigenic.[4] The results obtained could all be accounted for on this basis without the necessity of postulating a circulating resistance factor or antibody. It is evident that this difficulty is intrinsic in the method, since no matter how long the interval between immunization and parabiosis, active antigenic substances could still be circulating. It is interesting that in the one experiment of Bichel and Holm-Jensen in which the interval was long (43 days), no transfer of immunity was found. The authors explained this by assuming that the primarily inoculated animal had in that interval lost its own active immunity, but this assumption was not tested.

The method used in the experiments reported here serves, in some measure, to overcome the active immunity difficulty. Had the skins grafted to immune from nonimmunized mice become actively immunized, the grafts taken from immunized mice would presumably have shown some immunity in nonimmunized hosts.

There is another aspect of the Bichel and Holm-Jensen (*36*) experiments of the greatest importance. They showed quite conclusively that there is a vast difference between the results to be expected in cases of isologous, as opposed to homologous, parabiosis. By measuring the blood interchange between parabionts with the aid of "tagged" erythrocytes, they showed that in the particular genetic combination they used, interchange was 50 to 100 times greater in the isologous systems. Furthermore, it was only in isologous and not in homologous parabiosis that they were able to show a transfer of immunity. On the basis of this information, some of the contradictory earlier reports can be reconciled.

Since the data reported here support the hypothesis of a mobile or circulating resistance factor, it is incumbent to say a few words as to why it has often been difficult to demonstrate such a factor in the past. It seems that there are essentially three possible explanations: 1) The mobile factor does not circulate in the serum but is transported in association with a mobile cell such as the lymphocyte. 2) The mobile factor is not

[4] Despite the work of Barrett (*3*) with red cells, it cannot be assumed that only the intact cell is homologously antigenic when types of cells other than the red cell are used.

directly cytotoxic but represents only one link in a chain of resistance. 3) The mobile factor is usually present in such low titer in the serum that it is difficult to detect by the methods now available.

Experimental techniques can undoubtedly be devised to examine these possibilities, but at present there are no data to favor one or another of them.

Summary and Conclusions

The purpose of this paper is to offer experimental data giving support to the hypothesis of the existence of a mobile resistance factor in induced immunity to homologous tissue transplantation. In essence, the procedure in each experiment consisted of immunizing half of the experimental mice (which were all of the same inbred strain) to a particular transplantable mouse tumor. Approximately one month after immunization, skin grafts were exchanged between the immunized and nonimmunized animals. Four to five weeks following skin grafting, the grafts in both sets of mice were challenged (*i.e.* inoculated intradermally) with a fragment of the tumor. This procedure was repeated in three separate experiments employing genetically different systems.

The results were statistically highly significant. The tumor growths failed to exhibit any effects of an intragraft immunity in all series of nonimmunized mice, regardless of the source of the skin grafts. They showed the effects of immunity in all series of immunized mice, again regardless of the source of the skin grafts. These results support the generalization that a specific mobile resistance factor does exist in homologous tissue transplantation in the mouse. No sessile resistance factor could be detected by the use of the methods employed.

The implications of these findings were discussed in relation to the pertinent literature.

References

(*1*) PREHN, R. T.: Lack of intrinsic incompatibility between tissues of different genetic origin. J. Nat. Cancer Inst. 13: 873–881, 1953.

(*2*) MALMGREN, R. A., KLEIN, M., and PREHN, R. T.: Tumor tissue as a site for interstrain tumor transplantation in mice. J. Nat. Cancer Inst. 13: 883–888, 1953.

(*3*) BARRETT, M. K., HANSEN, W. H., and SPILMAN, B. F.: The nature of the antigen in induced resistance to tumors. Cancer Research 11: 930–936, 1951.

(*4*) ANDERVONT, H. B.: Studies on immunity induced by mouse sarcoma 180. Pub. Health Rep. 47: 1859–1877, 1932.

(*5*) PREHN, R. T.: Tumors and hyperplastic nodules in transplanted mammary glands. J. Nat. Cancer Inst. 13: 859–872, 1953.

(*6*) RANDALL, P., BROWN, J. B., and MCDOWELL, F.: An experimental method for the study of skin homografts. Surgical Forum; Proc. Clinical Cong. Am. Coll. Surg. (Phila.) 455–462, 1951.

(*7*) MANSFELD, G.: New pathways of the physiology of infection and immunity. Experientia 5: 188–199, 1949.

(*8*) KUCHERENKO, IU. G.: Skin homotransplantation on denervated area. Medichnii Zhurnal. Akademii U. R. S. R. 14: 283–285, 1945.

(9) SHEAR, M. J.: Chemical studies on tumor tissue III. Titration of mouse tumors. Pub. Health Rep. 51: 668–676, 1936.

(10) SCHREK, R.: The effect of the size of inoculum on the growth of transplantable rat tumors. Am. J. Cancer 28: 364–371, 1936.

(11) GORER, P. A.: Antibody response to tumor inoculation in mice with special reference to partial antibodies. Cancer Research 7: 634–641, 1947.

(12) ———: The role of antibodies in immunity to transplanted leukaemia in mice. J. Path. & Bact. 54: 51–65, 1942.

(13) KIDD, J. G.: Suppression of growth of Brown-Pearce tumor cells by a specific antibody, with a consideration of the nature of the reacting cell constituent. J. Exper. Med. 83: 227–250, 1946.

(14) ———: Suppression of growth of the Brown-Pearce tumor by a specific antibody. Science 99: 348–350, 1944.

(15) BURMESTER, B. R.: The cytotoxic effect of avian lymphoid tumor antiserum. Cancer Research 7: 459–467, 1947.

(16) LUMSDEN, T., and PHELPS, H. J.: Serological observations on spontaneous regression of implants of Jensen's rat sarcoma. Am. J. Cancer 29: 517–521, 1937.

(17) PHELPS, H. J.: A note on the non-specific action of so-called "anti-cancer serum." Am. J. Cancer 31: 441–445, 1937.

(18) TAKEDA, K.: Studies on the immunological phenomena of Yoshida sarcoma. Gann 43: 449–480, 1952.

(19) LAMBERT, R. A., and HANES, F. M.: A study of cancer immunity by the method of cultivating tissues outside the body. J. Exper. Med. 13: 505–510, 1911.

(20) GUSSIO, S.: Sul potere oncolitico nei ratti. Tumori 1: 75–86, 1911.

(21) MOTTRAM, J. C., and RUSS, S.: Observations and experiments on the susceptibility and immunity of rats towards Jensen's rat sarcoma. Proc. Roy. Soc. London, s.B., 90: 2–32, 1917.

(22) LEITCH, A.: Discussion on the present position of cancer research. Brit. Med. J. 2: 656–658, 1920.

(23) WEIL, R.: A study of the serum factor in tumor immunity. Proc. Am. Assoc. for Cancer Research, 9th Annual Meeting. J. Cancer Res. 1: 389–390, 1916.

(24) HARRIS, M.: The role of humoral antagonism in heteroplastic transplantation in mammals. J. Exper. Zool. 93: 131–145, 1943.

(25) LEWIS, M. R., APTEKMAN, P. M., and KING, H. D.: Inactivation of malignant tissue in tumor-immune rats. J. Immunol. 61: 321–326, 1949.

(26) MEDAWAR, P. B.: Tests by tissue culture methods on the nature of immunity to transplanted skin. Quart. J. Micr. Sc. 89: 239–252, 1948.

(27) ALLGOWER, M., BLOCKER, T. G., JR., and ENGLEY, B. W. D.: Some immunological aspects of auto- and homografts in rabbits, tested by in vivo and in vitro techniques. Plast. & Reconstruct. Surg. 9: 1–21, 1952.

(28) FINERTY, J. C.: Parabiosis in physiological studies. Physiol. Rev. 32: 277–302, 1952.

(29) KROSS, I.: Parabiosis and tumor growth. J. Cancer Research 6: 121–126, 1921.

(30) FURTH, O. B., BARNES, W. A., and BROWER, A. B.: Studies on resistance to transmissible leukemia in mice by means of parabiosis. Arch. Path. 29: 163–174, 1940.

(31) CLOUDMAN, A. M.: Reactions of hybrid and parabiontic pseudo-hybrid mice to inoculations of tumor C198. Cancer Research 3: 47–52, 1943.

(32) HARRIS, M.: The growth of alien strain tumors in parabiotic mice. Cancer Research 3: 546–549, 1943.

(33) BICHEL, J., and HOLM-JENSEN, I.: Parabiosis and resistance to transplantation III. Studies on the resistance to transmissible leukemia in mice by means of heterogenetic and homogenetic parabiosis. Acta path. et microbiol. scandinav. 26: 319–325, 1949.

(*34*) Taylor, M. J., and MacDowell, E. C.: Mouse leukemia XIV. Freeing transplanted line I from a contaminating virus. Cancer Research 9: 144–149, 1949.

(*35*) Law, L. W., and Dunn, T. B.: Effects of a filtrable, self-propagating contaminant on a transplantable acute lymphoid leukemia in mice. J. Nat. Cancer Inst. 11: 1037–1055, 1951.

(*36*) Bichel, J., and Holm-Jensen I.: Determination by means of radioactive blood corpuscles of the crossed blood flow between parabiotic mice. Acta physiol. Scandinav. 17: 255–260, 1949.

On the Factor of Age in the Production of Subcutaneous Sarcomas in Mice by 20-Methylcholanthrene [1]

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The reaction induced by subcutaneously injected carcinogens is modified by a number of factors including age variation (*1*, *2*). The conclusions reached regarding the importance of age in carcinogenesis are not in agreement, and some investigators believe that it is of no significance (*3*, *4*). In one study (*4*) the latent period prior to development of a tumor appeared to be greater in young animals. Shimkin and Bryan (*5*) and Strong *et al.* (*6*) found that tumors developed earlier and in higher percentage in young animals than in old ones. The present paper deals with the experimental production of sarcomas in mice of different ages.

Materials and Methods

Male mice of strain C3H were used. The carcinogen was administered as a single subcutaneous injection in the right axillary region. The age groups were: *a*) 1- to 2-week-old mice, *b*) 3-month-old mice, and *c*) 1-year-old mice. In one experiment a dose of 0.03 mg. of methylcholanthrene dissolved in 0.2 cc. of tricaprylin was injected into mice of the three groups. In a second experiment, a dose-weight ratio was employed using the same concentration of the carcinogen, so that the first group of mice (1 to 2 weeks old) received 0.0045 mg. of methylcholanthrene and the second group (3 months old) received 0.225 mg. of methylcholanthrene (no 1-year-old mice being treated). The mice were examined twice a week.

The animals were killed with ether and the tissues fixed in Zenker's formol fluid. The tissues were embedded in paraffin and the sections prepared with hematoxylin and eosin, van Gieson's, Masson's trichrome, and Wilder's silver impregnation methods, the periodic acid-Schiff reaction, and the phosphotungstic acid–hematoxylin stain. Studies were made of 147 primary tumors and 41 transplanted tumors.

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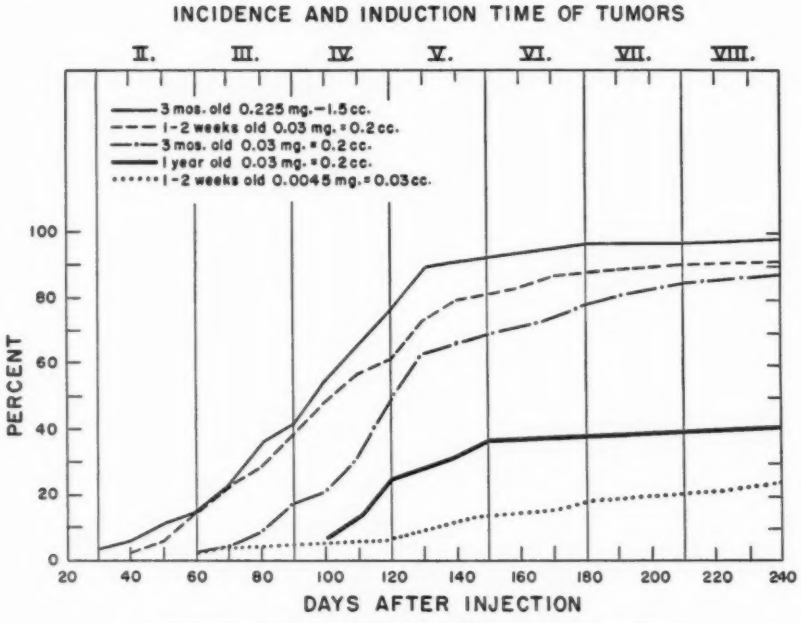

TEXT-FIGURE 1.—Incidence and induction time of tumors.

Results

The results are tabulated in table 1 and presented graphically in text-figures 1 and 2.

Incidence of tumors.—The same amount of carcinogen produced the same number of tumors in mice 1 to 2 weeks old as in mice 3 months old (91.8 and 92.7 percent respectively), but in 1-year-old mice the incidence of tumors was only 43.7 percent. When the dose-weight ratio was employed the tumor incidence was much lower (26.5 percent) in mice 1 to 2 weeks old receiving 0.0045 mg. of methylcholanthrene than it was in 3-month-old mice receiving 0.03 mg. of methylcholanthrene (92.7 percent). The incidence of tumors in mice 3 months old receiving larger doses (0.225 mg. of methylcholanthrene) was 97.2 percent, corresponding to 91.8 percent in mice 1 to 2 weeks old receiving 0.03 mg. of the carcinogen.

Incidence of different types of tumors.—Most of the myoblastic sarcomas were found in mice injected at 1 to 2 weeks of age. In this group, the myoblastic sarcomas constituted 41 percent of the induced tumors, whereas in mice 3 months of age receiving the same dose only 8.8 percent of the tumors were myoblastic sarcomas, and in mice 1 year old only 7.1 percent.

About half of the induced tumors in all experimental groups were rhabdomyosarcomas. In the year-old mice 42.9 percent of the induced tumors were spindle-cell sarcomas, while in mice 3 months of age 32.4 percent were spindle-cell tumors, and only 14.5 percent were spindle-cell type in mice 1 to 2 weeks old.

TABLE 1.—*The effect of subcutaneously administered 20-methylcholanthrene in mice of different ages*

Experimental group and doses administered	Age of mice	No. of mice	Number of tumors	Incidence (*percent*)	Induction time (mean and range) (*days*)	Growth rate* (mean and range)	Types of tumors			Induction time for different types of tumors (mean and range)		
							Myoblastic sarcomas (*percent*)	Rhabdomyosarcomas (*percent*)	Round-cell and spindle-cell sarcomas (*percent*)	Myoblastic sarcomas (*days*)	Rhabdomyosarcomas (*days*)	Round-cell and spindle-cell sarcomas (*days*)
0.03 mg. of methylcholanthrene dissolved in 0.2 cc. tricaprylin	1–2 wks.	49	45	91.8	107 (40–250)	63.1 (52–78)	41.1	44.4	14.5	88 (40–250)	117 (70–170)	124 (70–190)
	3 mos.	55	51	92.7	140 (60–360)	63.7 (44–73)	8.8	58.8	32.4	94 (60–190)	132 (80–350)	168 (80–360)
	1 yr.	16	7	43.7	147 (100–290)	38.2 (36–43)	7.1	50	42.9	120	166 (100–290)	125 (100–150)
0.0045 mg. of methylcholanthrene dissolved in 0.03 cc. tricaprylin	1–2 wks.	34	9	26.5	168 (70–250)	46.3 (40–63)	11	50	39	70	156 (120–220)	210 (120–250)
0.225 mg. of methylcholanthrene dissolved in 1.5 cc. tricaprylin	3 mos.	36	35	97.2	95 (30–180)	65.4 (56–78)	14.3	67.1	18.6	91 (50–180)	100 (30–180)	112 (70–160)

*Growth rate $K=\frac{\log D_2-\log D_1}{t_2-t_1}$. ($D_2$ is the mean diameter at time t_2, and D_1 the mean diameter at time t_1.

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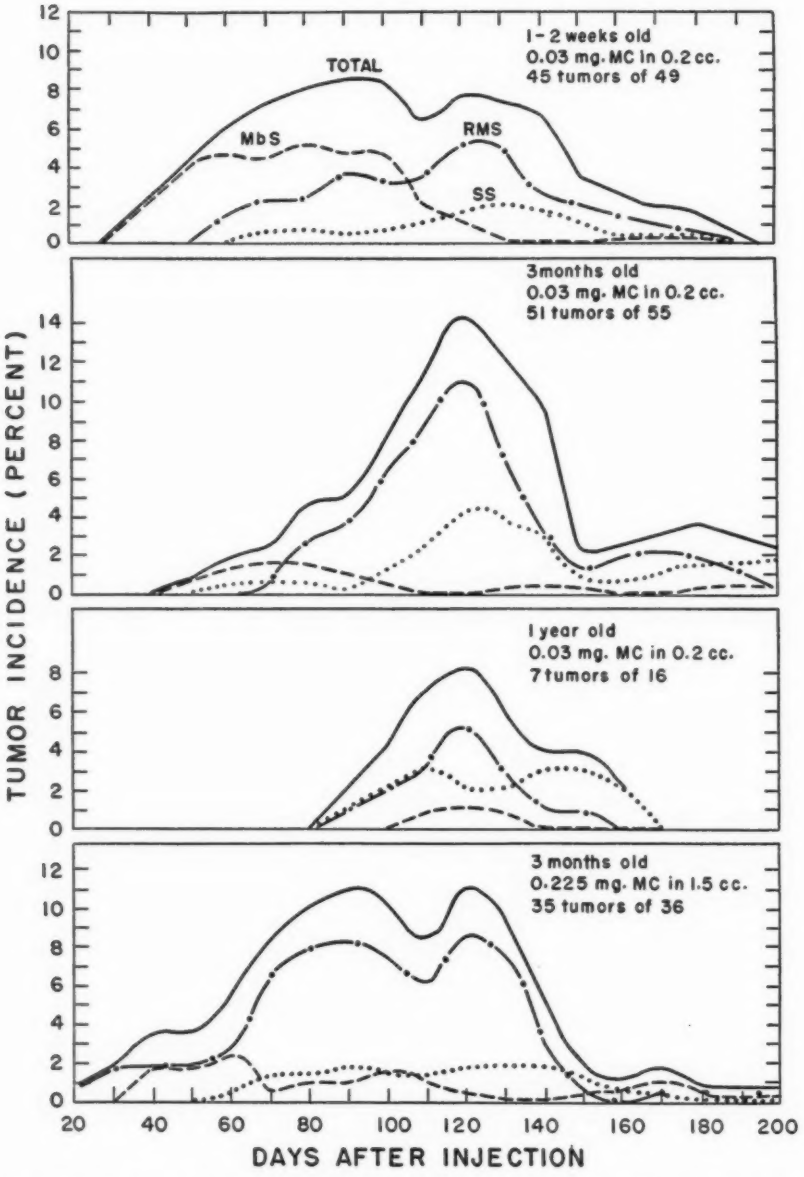

TEXT-FIGURE 2.—Induction time of various types of tumors in different groups of mice. MbS=myoblastic sarcoma. RMS=rhabdomyosarcoma. SS=spindle-cell and round-cell sarcomas.

The 3-month-old mice that received the larger doses of carcinogen showed a slightly higher incidence (14.3 and 67 percent) of myoblastic sarcomas and rhabdomyosarcomas than did mice of the same age given lower doses (8.8 and 58.8 percent). In the group of mice 1 to 2 weeks old that received the smaller dose, 9 tumors were induced and only one of these was a myoblastic sarcoma.

Induction time of tumors.—The induction time of tumors varied from 30 to 360 days. When using the same amount of carcinogen for different ages, the mean induction time for tumors in 1-year-old mice and 3-month-old mice was about the same (147 and 140 days), but in mice 1 to 2 weeks old it was only 107 days. The time required to obtain 50 percent incidence of tumors in the older age groups was 117 and 118 days. Among the mice 1 to 2 weeks old, 50 percent of the animals developed tumors 20 days earlier (at 97 days). When the dose-weight ratio was employed, the induction time in 1- to 2-week-old mice receiving only 0.0045 mg. of the carcinogen was found to range from 70 to 250 days (mean 168), corresponding to a mean of 140 days in 3-month-old mice receiving 0.03 mg. of the carcinogen. When the 3-month-old mice received a larger dose (0.225 mg.), the mean induction time was shortened from 140 to 95 days, corresponding to 107 days in mice 1 to 2 weeks of age receiving 0.03 mg. of carcinogen.

Induction times for different types of tumors.—In all experimental groups, the induction time for myoblastic sarcomas was shorter than that for the other tumor types (text-fig. 2). However, the variation within any one experimental group was considerable. Thus, in the mice 1 to 2 weeks old, the first myoblastic sarcoma appeared 40 days after injection, and the last tumor at 250 days after injection of the carcinogen. Most of the myoblastic sarcomas appeared between 2 and 3 months after the injection. The peak of the incidence of rhabdomyosarcomas was 4 months after the injection, and that of spindle-cell and round-cell sarcomas usually a little later. It is interesting to note that the induction time of the same type of tumor did not vary significantly in the different experimental groups. The greatest difference in the different age groups was, therefore, not in the induction time of tumors, but in the incidence of the different types of tumors.

Histology of induced tumors.—The histology of the induced sarcomas varied greatly. Many tumors were composed principally of spindle cells and round cells, while others appeared to consist chiefly of modified skeletal muscle fibers, muscle giant cells, and myoblasts. The tumors were separated, according to the type of tissue comprising the greater part of the growth, into three groups: 1) myoblastic sarcomas, 2) rhabdomyosarcomas, and 3) spindle-cell and round-cell sarcomas.

Myoblastic Sarcomas

The myoblastic sarcomas (figs. 1 to 5) were always adherent to striated muscle and revealed evidence of proliferation of the nuclei of cells resembling muscle fibers. These were distinguished from the nuclei of sar-

colemma by the smaller amount of chromatin they contained, and they regularly showed one or two distinct nucleoli. No mitotic figures were found inside the old sarcolemma. The nuclei were arranged in rows, closely packed together, and deeply embedded in the striated fibers. The nucleus was surrounded by a zone of faintly stained cytoplasm in which no striation could be seen. This perinuclear zone had a purplish blue tint in hematoxylin-eosin preparations, in striking contrast to the eosinophilic muscle substance of the unaltered areas and of the degenerating muscle fibers. In sections treated with Masson's trichrome stain, the zone appeared reddish blue in contrast to the red color of normal muscle fibers. Some of these tumors, therefore, resembled hyperplastic muscle regeneration. Most of the tumors, however, were composed of large round cells with homogeneous or finely granular cytoplasm and a delicate cell membrane. These round cells usually possessed a single nucleus, although some of the larger cells contained two or three nuclei. The nuclei were large and vesicular, and contained a single very prominent nucleolus. Evidence was found which seems to indicate that these large round cells called myoblasts may have been derived from regenerating muscle fibers.

Some of the tumors were pleomorphic with respect to cell types, and mitotic figures were numerous. The nuclei were darkly stained, and the cytoplasm less abundant. A single, deeply basophilic nucleolus was a prominent feature of these cells and some smaller basophilic masses were usually present in the nucleus. The cytoplasm was negative to the periodic acid-Schiff reaction, and no cross striation was observed. These pleomorphic cells resembled very closely the myoblasts seen in other parts of the tumor, both in morphology and tinctorial properties. The reticulum was scarce and surrounded single cells and groups of cells. Tumors of this group have been subdivided into uniform and pleomorphic myoblastic sarcomas.

Rhabdomyosarcomas

The histologic picture of these tumors (figs. 6, 7) was usually very different from that of the tumors called myoblastic sarcomas. The large rounded cells with distinct cell membranes were lacking. The tumors were made up of large spindle-shaped cells, extremely variable in size and shape and, in some areas, arranged in conspicuous bands. Scattered giant cells were found, which were round, oval or straplike (fig. 6). They had a relatively large amount of cytoplasm, which was homogeneous or finely granular. Unequivocal cross striation was not observed in any of the tumors, but fine longitudinal myofibrils were seen in some. In addition to the giant cells and large spindle cells, small cells with dark round or oval nuclei were also found (fig. 7). The stroma was inconspicuous and the tissue consisted almost entirely of tumor cells, between which were a few thin-walled blood vessels. The reticulum was coarse and surrounded larger cell groups between which no reticulum could be seen. The tumors were diagnosed as rhabdomyosarcomas.

Spindle-Cell and Round-Cell Sarcomas

No myoblasts or skeletal muscle elements were found in these tumors (figs. 8 to 13). The tumors were composed of pleomorphic round cells or spindle cells arranged in conspicuous bands and interlacing bundles. The nuclei were usually smaller than in the preceding groups and mitoses were numerous. The collagenic material varied in amount but was usually scanty. A few tumors, however, were rich in collagenic fibers, in many of which fibroglia could be demonstrated by the phosphotungstic acid–hematoxylin stain. This seemed to indicate that these tumors belonged to the fibrosarcomas. In the majority of tumors, no fibroglia could be demonstrated. The reticulum fibers varied in thickness and formed a meshwork embracing cell groups and single cells. These spindle-cell and round-cell sarcomas comprise a heterogeneous group.

Transplantation experiments.—(Figs. 14, 15.) All of the 8 primary tumors transplanted in mice of the strain of origin grew successfully. Of these primary tumors, 4 were myoblastic sarcomas, 3 rhabdomyosarcomas, and 1 a spindle-cell sarcoma. The 35 first-generation transplants and 6 second-generation transplants were studied. Of the first-generation transplants, 10 had been transplanted in mice 1 to 2 weeks old, 18 in 3-month-old mice, and 7 in 1-year-old mice. All of the second-generation transplants were placed in 3-month-old hosts, and all were derived from a primary myoblastic sarcoma induced in a 2-week-old mouse and transplanted to a 3-month-old mouse.

In all cases the transplants had the same histologic pattern as the primary tumor. The age of the host did not modify the morphology of the transplanted tumor, and the sections from tumors transplanted subcutaneously and intraperitoneally were similar. When a suspension of tumor cells or a piece of tumor was used for transplantation, the tumors also had the same histologic pattern.

Discussion

In the present study, significant variations were noted in the incidence, induction time, and histology of tumors induced in mice of different ages by subcutaneous injection of methylcholanthrene.

Young mice seem to be more susceptible to the induction of tumors with a given dose of carcinogen than do older mice, and myoblastic sarcomas are more commonly induced in young mice. Before assuming that the cells of a younger animal respond to carcinogen in a different way than cells of an older animal, many other possible factors should be considered.

When the same dose, *i.e.* the same amount of carcinogen in the same concentration, is injected into mice of different age, it is logical to assume that the number of cells that come into direct contact with the carcinogen is the same at all ages. However, in young and small mice, the amount of carcinogen is much greater per gram of body weight of the mouse than it is in older and larger mice. It should also be noted that even if the

number of cells that come into contact with the carcinogen were the same in young and old animals, the types of cells may be different, and thus the histogenesis of induced tumors may vary.

More cells may be transformed into tumor cells if the carcinogen stays longer at the injection site, and the possibility of affecting a cell is greater. This would be the case if the *rate of elimination* of the carcinogen were different in different ages. No reports of any investigation of this point have been found.

It has been shown that if the mean induction time be plotted versus the reciprocal concentration, the resulting curve is a straight line (*7*, *5*). It has also been shown that the induction time is directly proportional to the length of time the methylcholanthrene-cholesterol pellets are permitted to remain *in situ* (*8*). In the present study, the higher incidence and the shorter induction time noted in younger animals could thus well be the result of a longer elimination period of the carcinogen in younger animals. This assumption is supported by the fact that tumors in mice 1 to 2 weeks old were more evenly distributed in relation to the induction time, and by the fact that this was also the case when the larger dose was used for the 3-month-old mice. This explanation, however, hardly explains the differences noted between the 3-month-old mice and the 1-year-old mice.

That the rate of elimination of the carcinogen is not the only important factor is shown by the studies of Weil-Malherbe (*9*, *10*), who found that by using benzpyrene-tricaprylin solution the rate of elimination of benzpyrene is accelerated when the solvent contains cholesterol, and that this is associated with a higher carcinogenic activity. When the solvent contained phospholipid, the rate of elimination was increased and the carcinogenic activity diminished.

In the present study it was noted that the induction time of myoblastic sarcomas in all experimental groups was significantly shorter than that of tumors of the other types. It was also found that the induction time of the different types of tumors did not vary significantly in the different experimental groups. Taking into account also that the proportion of myoblastic sarcomas in the group of mice 1 to 2 weeks old was much greater than in the older age groups when the same dose was used, we may say that the main difference in response to carcinogen between the mice 1 to 2 weeks of age and the mice 3 months of age was not in the incidence of tumors and not in the induction time of tumors, but in the histology of the induced tumors. The proportion of different types of tumors was almost the same in 3-month-old mice and 1-year-old mice, and thus the main difference between these age groups was in the incidence of tumors.

When dose-weight ratio was employed, increasing the dose for 3-month-old mice, the over-all incidence was about the same but the frequency of different tumor types was different in that the relative number of tumors with muscle elements was increased. When use of the dose-weight ratio decreased the dose for mice 1 to 2 weeks old, only 9 tumors (27

percent) were induced and only one of these was a myoblastic sarcoma. Thus, it seems as if the frequency of different tumors was not so much due to the age of the animal as to the amount of carcinogen used.

Histogenesis of the induced tumors.—From the microscopic study of the induced tumors it becomes evident that at least three different types of tumors were produced. In two of these types muscle elements were present, in the third they were lacking. The question arises, whether the muscle elements represent the early reaction of muscle to injury by the carcinogen or represent only the early regeneration of skeletal muscle. Whether or not these tumors are true muscle tumors with, perhaps, a malignant dedifferentiation of the affected muscle elements into spindle cells and round cells is also open to question. Speculations have been published for (*11*) and against (*12*) the latter possibility.

Many sarcomas were found in the present series in which muscle elements formed the greatest part of the tumor. Areas were seen where the picture was that of a very active muscle regeneration (fig. 4), marked proliferation of muscle nuclei inside the old sarcolemma (figs. 16, 17), and formation of myoblasts with distinct cell limits and usually one (but occasionally two or three) nuclei. This picture changed gradually into areas where the size of the cells varied, nuclear polymorphism was seen, and atypical mitoses were found. There was, therefore, an unusually active and excessive muscle regeneration which extended into the adjacent tissues. It is impossible to prove a direct connection between the pleomorphic round cells and the myoblasts, but the appearance suggests that the muscle cells could be dedifferentiating into malignant round cells and spindle cells.

In normal muscle regeneration (*13*) the nuclei within the newly formed muscle cells multiply, and when there are 2 to 6 nuclei in the cells, the cytoplasm begins to differentiate and fine longitudinal striation can be seen. In two tumors some maturation was suggested by the formation of fine longitudinal fibrils and by the greater length of the cells (fig. 18). In most tumors nothing indicating differentiation could be observed and the whole picture appeared to be formation of myoblasts and "dedifferentiation" of these into pleomorphic round cells and spindle-shaped cells.

The histology of the primary tumors diagnosed as myoblastic sarcomas is thus easily explained by regarding them as true muscle tumors. However, no final proof can be obtained, and the so-called "transitional forms" may be artifacts. It is also known that the invaded tissues are sometimes stimulated into a proliferation that may be indistinguishable from neoplasia (*14*).

In the transplantation experiments it was found that the first- and second-generation transplants were all similar to the primary tumor. The histology of the transplant was not influenced by the age of the host. Thus, the histology of the transplanted tumors may be taken as supporting the muscle origin of the primary tumor. There are, however, other explanations as well. The muscle elements in transplanted tumors may be derived from the host muscle or from regenerating muscle fibers that

were transplanted with the tumor. The possibility that the tumor suspension contained sufficient carcinogen to produce regeneration or neoplasia of the host muscle is minimal.

Against the possibility of collateral hyperplasia of muscle invaded by the transplanted tumor is the fact that myoblast-like cells were found also in tumors transplanted into the peritoneal cavity. It should also be pointed out that tumors of the other types, when transplanted, never induced collateral hyperplasia of invaded muscle. Therefore, it is logical to assume that the muscle elements found in transplanted tumors have originated from the transplanted cells.

Summary and Conclusions

One hundred forty-seven sarcomas were produced following subcutaneous injection of 20-methylcholanthrene into mice of three different age groups: 1 to 2 weeks, 3 months, and 1 year.

The difference found between mice 3 months and 1 year of age in response to carcinogen is chiefly in the incidence of tumors. The incidence is lower in year-old mice, indicating that the tissues of a senescent animal are less susceptible to malignant transformation than the tissues of fully developed younger animals. The frequency of the different histologic types of tumors is about the same.

The main difference between mice 1 to 2 weeks old and mice 3 months of age is in the histology of induced tumors. When the same dose of carcinogen is used in the very young mice, either more tumors of true myoblastic origin or more tumors with a definite myoblastic proliferation are induced.

The histologic differences found are not, however, necessarily due to the age of the animal, for it cannot be concluded without reservation that the subcutaneous tissue of a very young and rapidly growing animal is more prone to sarcomatous transformation after methylcholanthrene injection than is the subcutaneous tissue of fully developed animals. When the same amount of carcinogen is injected into a small animal, more muscle tissue comes into direct contact with the carcinogen than in an older animal. Therefore, the greater number of tumors of muscle origin or of tumors with a strong myoblastic proliferation in the young mice may be explained by the greater amount of regenerating muscle tissue that comes into contact with the carcinogen.

References

(*1*) SHIMKIN, M. B.: Factors influencing carcinogenesis with methylcholanthrene. I. The effect of age. Pub. Health Rep. 54: 1388–1391, 1939.

(*2*) STEWART, H. L.: Tumors induced by subcutaneously injected carcinogens. Bull. Hosp. Joint Dis. 12: 131–147, 1951.

(*3*) BRUNSCHWIG, A., and TSCHETTER, D.: Age factor and latent period in production of sarcoma by methylcholanthrene in rats. Proc. Soc. Exper. Biol. & Med. 36: 439–441, 1937.

(4) DUNNING, W. F., CURTIS, M. R., and BULLOCK, F. D.: Respective roles of heredity and somatic mutation in origin of malignancy. Am. J. Cancer 28: 681–712, 1936.

(5) SHIMKIN, M. B., and BRYAN, W. R.: Morphology and growth of subcutaneous tumors induced with carcinogenic hydrocarbons in strain C3H male mice. J. Nat. Cancer Inst. 4: 25–35, 1943.

(6) STRONG, L. C., SMITH, G. M., and GARDNER, W. U.: Induction of tumors by 3:4:5:6-dibenzcarbazole in male mice of CBA strain, which develops spontaneous hepatomas. Yale J. Biol. & Med. 10: 335–346, 1938.

(7) ENGELBRETH-HOLM, J., and IVERSEN, S.: On the mechanism of experimental carcinogenesis. II. The effect of different concentration of 9,10-dimethyl-1,2-benzanthracene on skin-carcinogenesis in mice. Acta path. et microbiol. scandinav. 29: 77–83, 1951.

(8) ANDERVONT, H. B., and SHEAR, M. J.: Production of tumors in mice following removal of methylcholanthrene cholesterol pellets. J. Nat. Cancer Inst. 2: 333–334, 1942.

(9) WEIL-MALHERBE, H., and DICKENS, F.: Factors affecting carcinogenesis. IV. The effect of tricaprylin solutions of cholesterol and phospholipins. Cancer Research 6: 171–178, 1946.

(10) WEIL-MALHERBE, H., The elimination and carcinogenic potency of 3:4-benzpyrene in mice after subcutaneous injection of non-lipoid solutions. Brit. J. Cancer 1: 423–431, 1947.

(11) HAAGENSEN, C. D., and KREHBIEL, O. F.: The morphology of sarcomas produced by 1:2:5:6-dibenzanthracene. Am. J. Cancer 26: 368–377, 1936.

(12) LEWIS, W. H.: Dibenzanthracene mouse sarcomas; histology. Am. J. Cancer 37: 521–530, 1939.

(13) FORBUS, W. D.: Pathologic changes in voluntary muscle. II. Experimental study of degeneration and regeneration of striated muscle with vital stains. Arch. Path. & Lab. Med. 2: 486–499, 1926.

(14) FOULDS, L.: The histological analysis of tumours. A critical review. Am. J. Cancer 39: 1–24, 1940.

PLATE 38

FIGURE 1.—Uniform myoblastic sarcoma infiltrating below the epidermis. Hematoxylin and eosin. × 190

FIGURE 2.—Same tumor as in figure 1. Reticulum shown by Wilder's silver impregnation method. × 500

FIGURE 3.—Same tumor as in figures 1 and 2. High-power view of large cells resembling myoblasts. Hematoxylin and eosin. × 500

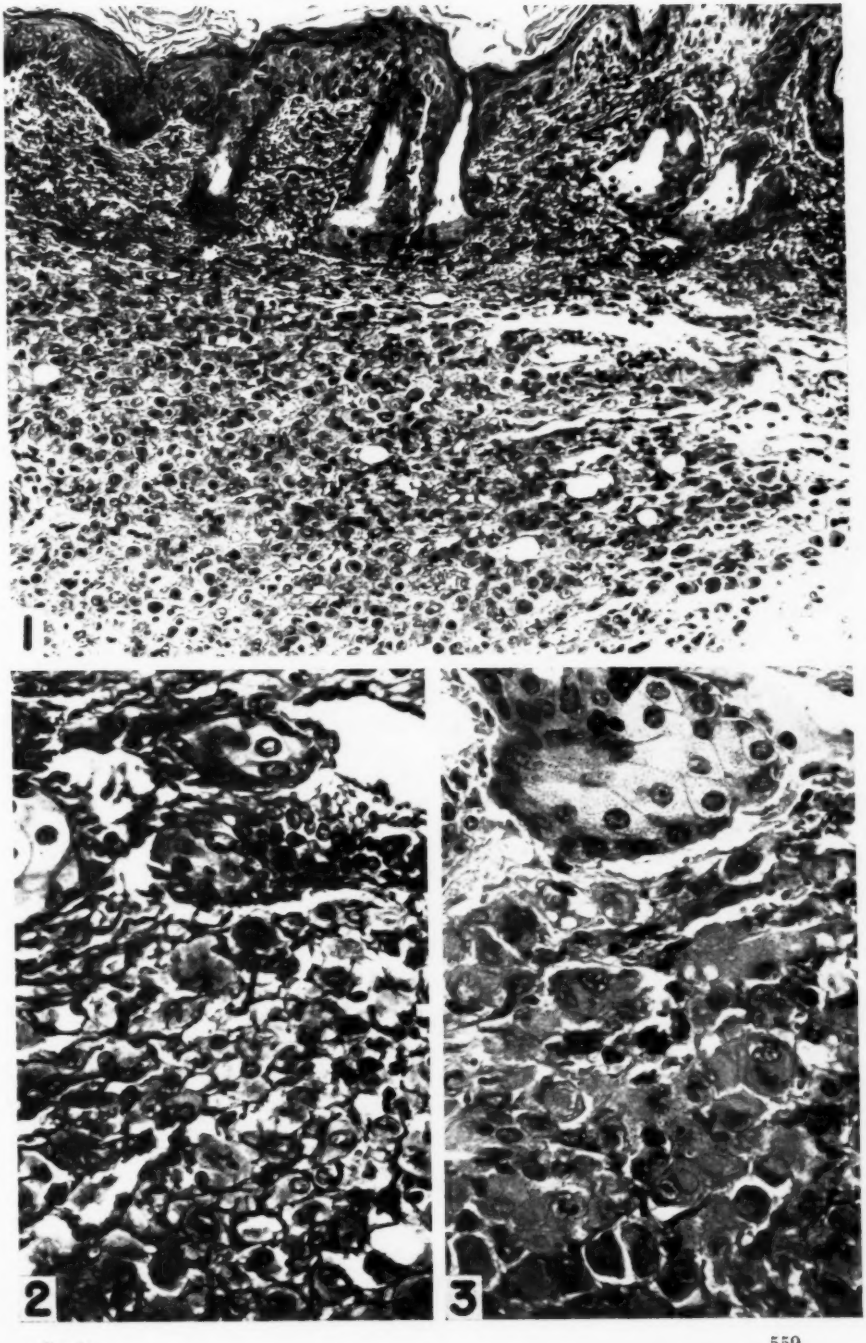

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PLATE 39

FIGURE 4.—Pleomorphic myoblastic sarcoma. Proliferation of muscle nuclei and pleomorphic round cells resembling myoblasts. Hematoxylin and eosin. × 500

FIGURE 5.—Pleomorphic myoblastic sarcoma. An area with many mitotic figures and marked polymorphism of cells. Hematoxylin and eosin. × 610

FIGURE 6.—Rhabdomyosarcoma. Giant cells and pleomorphic spindle cells. Hematoxylin and eosin. × 610

FIGURE 7.—Rhabdomyosarcoma. Pleomorphic spindle cells with prominent nucleoli and smaller spindle-shaped cells with hyperchromatic nuclei. Hematoxylin and eosin. × 610

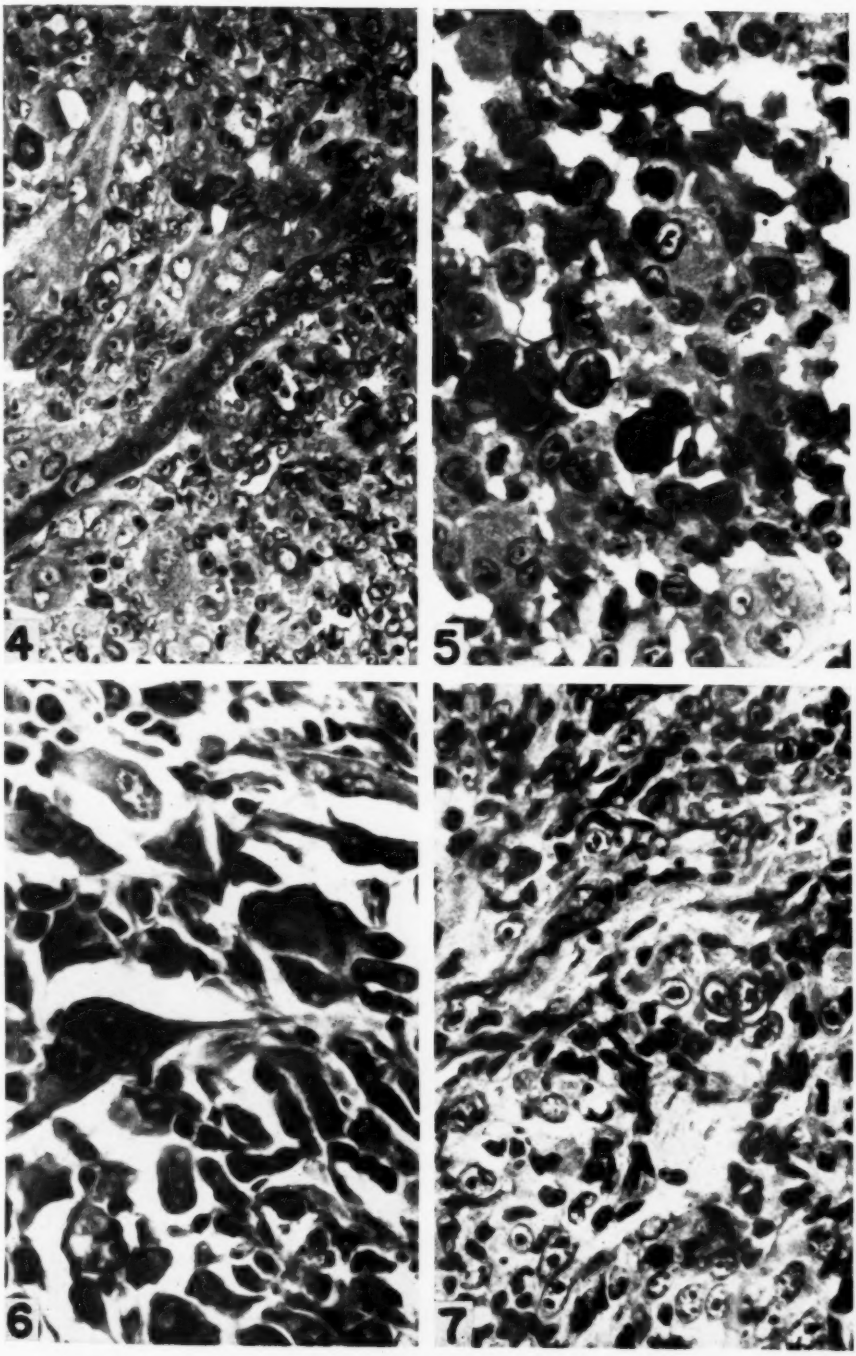
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PLATE 40

FIGURE 8.—Spindle-cell sarcoma of probable muscle origin. Hematoxylin and eosin. × 610

FIGURE 9.—Reticulum of a spindle-cell sarcoma. The reticulum suggests a muscle origin. Wilder's silver impregnation method. × 260

FIGURE 10.—Round-cell sarcoma. The myoblastic origin is probable. Hematoxylin and eosin. × 500

FIGURE 11.—Reticulum of the same tumor shown in figure 10. Wilder's silver impregnation method. × 500

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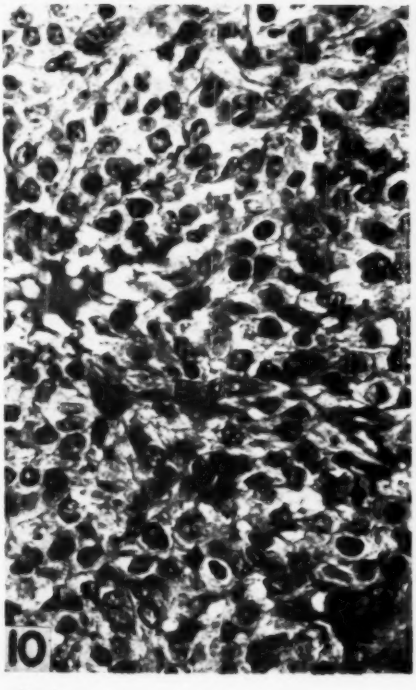
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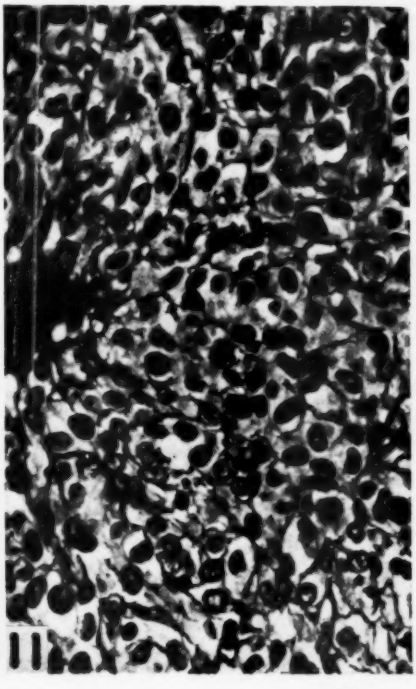
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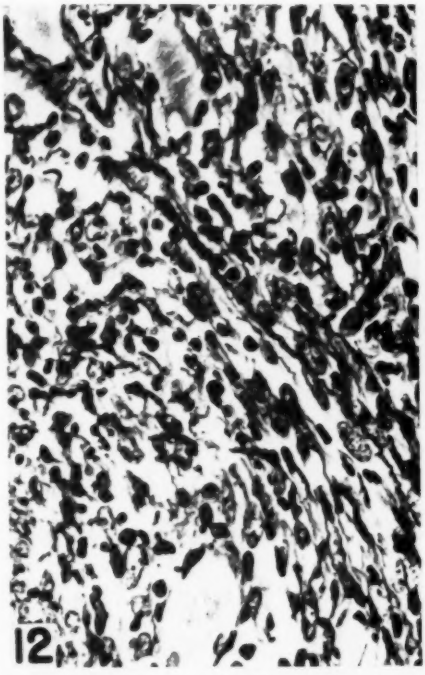
PLATE 41

FIGURE 12.—Reticulum of a spindle-cell sarcoma that is infiltrating muscle. Wilder's silver impregnation method. × 500

FIGURE 13.—Round-cell sarcoma infiltrating muscle. Hematoxylin and eosin. × 610

FIGURE 14.—Rhabdomyosarcomatous area in myoblastic sarcoma transplanted into the abdominal cavity. Hematoxylin and eosin. × 500

FIGURE 15.—Myoblastic sarcoma transplanted into the abdominal cavity. Hematoxylin and eosin. × 500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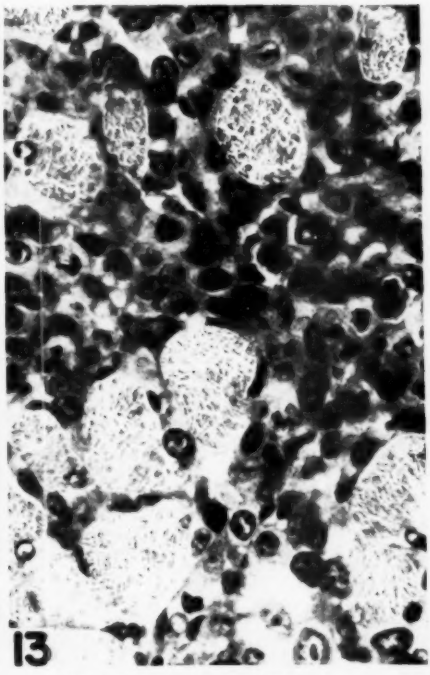
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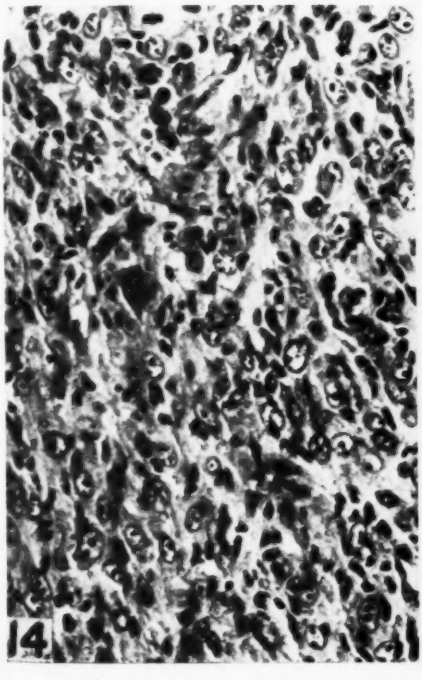
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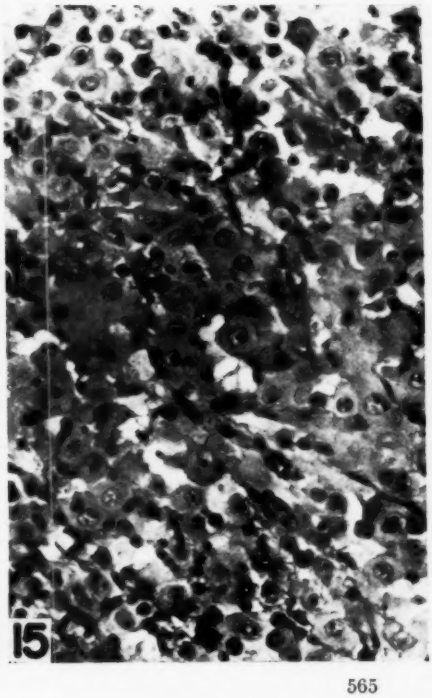
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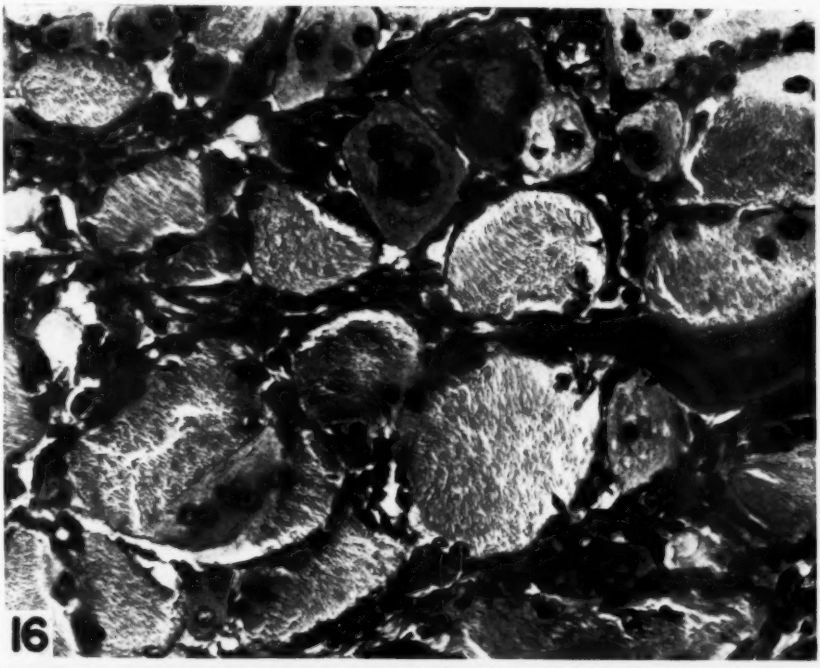
PLATE 42

FIGURE 16.—Nuclear proliferation inside the old sarcolemma and formation of muscle cells. Hematoxylin and eosin. × 500

FIGURE 17.—Very active muscle regeneration and pleomorphic round cells resembling myoblasts. Hematoxylin and eosin. × 500



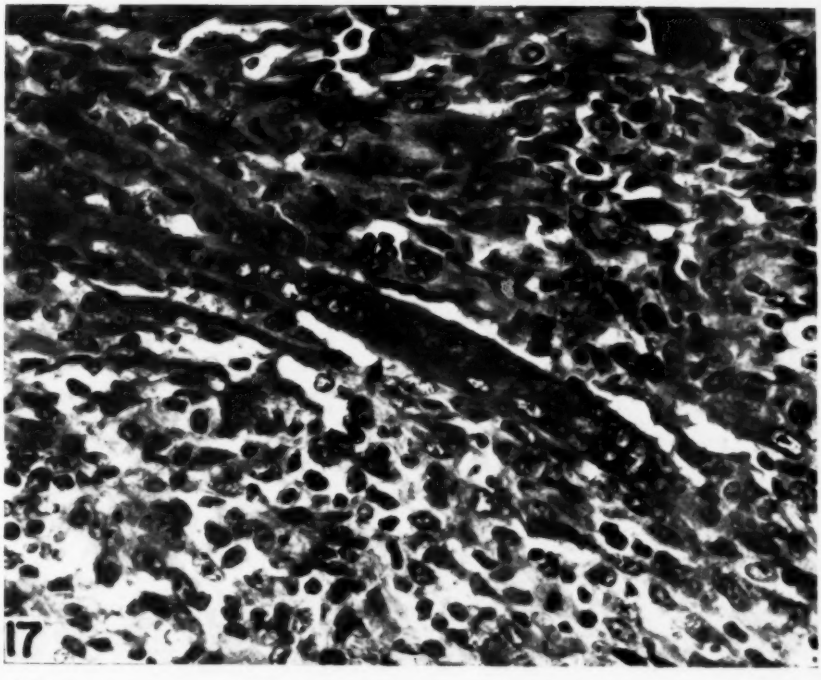


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PLATE 43

FIGURE 18.—Neoplastic muscle regeneration and formation of primitive spindle cells. Some maturation is indicated by the formation of fine longitudinal muscle fibrils and by the considerable length of the cells. Hematoxylin and eosin. × 500

FIGURE 19.—Muscle regeneration. Hematoxylin and eosin. × 610

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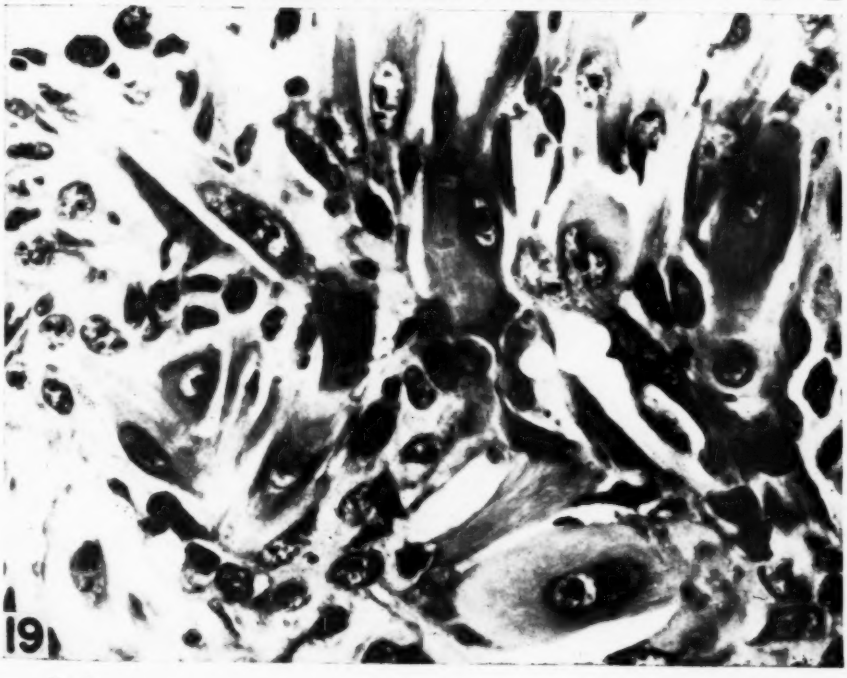
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Induction of Liver Tumors in Rats Fed *p*-Dimethylaminobenzene-1-Azo-2-Naphthalene [1]

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Tumors of the liver, forestomach, and skin have been produced in rats fed *p*-dimethylaminobenzene-1-azo-1-naphthalene (DAN) by Mulay and Firminger (*1*, *2*). The related β-azonaphthalene compound *p*-dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N) produced hemangioendotheliomas of the skin and liver tumors when painted on C57BR mice (*3*). Kinosita (*4*) failed to produce tumors in rats fed DA-2-N.

This paper reports the successful induction of liver tumors in Osborne-Mendel rats by feeding DA-2-N, and the failure to produce tumors by painting the skin of the same strain of rats with the same azo compound.

Materials and Methods

The feeding experiment.—Fifty-one Osborne-Mendel rats, 3 to 4 months old, 35 males and 16 females, were fed a semisynthetic diet containing 0.075 gm. of DA-2-N per 100 gm. of food, for 230 days. The animals were returned to a diet of Purina laboratory chow pellets at the end of this period. The composition of the semisynthetic diet and the method of incorporating a carcinogen into it were reported previously (*1*).

Beginning 5 months after the start of the experiment, a few animals were sacrificed every month, so that tissues were available for study of progressive changes. All animals were examined biweekly for palpable tumors and the date recorded when a tumor was first noted. When an animal appeared sick or had a large tumor it was selected for autopsy. The last animal was sacrificed 16 months after the start of the experiment. The icterus index and serum bilirubin were determined on every animal just before autopsy.

The gross diagnosis of tumor was confirmed by histologic study in every animal. A histologic section was examined from each conspicuous tumor and many more sections were examined from nontumorous parts of each liver. When a tumor was very large, several sections from different areas were taken. No attempt was made to take sections from every small tumor. Sections were also examined from the lungs, spleen,

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stomach, kidneys, adrenal glands, pancreas, and gonads. When observed, metastatic masses were always taken for microscopic study. The tissues were fixed in 10 percent formalin, Zenker's acetic fluid or Zenker's formol. Microscopic sections were routinely stained with hematoxylin and eosin.

Skin-painting experiment.—Ten Osborne-Mendel rats (5 males and 5 females) were painted twice a week for 18 months with a 1-percent solution of DA-2-N in benzene. Assuming that about 0.5 ml. of the solution was painted on the skin at each application, each rat received a total calculated dose of about 780 mg. of the azo dye. These rats were kept on a regular laboratory diet of Purina laboratory chow, and examined periodically for skin lesions. At the end of the experimental period all the animals were sacrificed. The skin at the site of the painting and the liver were examined histologically. Tissues that were examined and the method of preparation were the same as in the feeding experiment.

Results

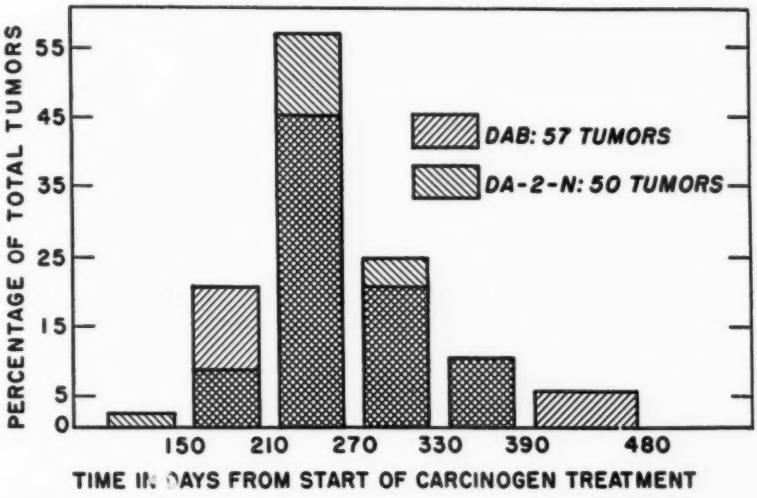
Lesions of the rats fed DA-2-N.—The types of hepatic lesions produced and their frequencies, in the rats fed DA-2-N, have been tabulated in table 1.

TABLE 1.—*Types of tumors induced by* p-*dimethylaminobenzene-1-azo-2-naphthalene* (*DA-2-N*) *compared with those induced by* p-*dimethylaminoazobenzene* (*DAB*) *or* p-*dimethylaminobenzene-1-azo-1-naphthalene* (*DAN*) (*1*). *Number of rats with the lesion per 100 rats surviving the induction period is recorded.*

Lesion	DA-2-N	DAN	DAB
Hepatoma	74	72	28
Cholangiocarcinoma	12	17	35
Cholangiohepatoma	66	15	67
Undifferentiated	10	17	....
Metastases to lungs	62	42	39
Number of peritoneal implant sites*	148	11	37

*All the implants on one organ are counted as one implant site. Mean time lapse between the first palpation of a liver tumor and the sacrifice of that rat was about the same in each group of animals. However, all the figures in this table are viewed as possible trends and not as quantative differences in the behavior of the carcinogens used.

The first rat died 18 days after the start of the feeding experiment. It had no evidence of a liver tumor. A second rat that died at 59 days showed several small tumors. All the remaining 49 rats developed palpable tumors which were discovered on periodic examination and confirmed at autopsy. Four of the 49 rats had clinically palpable tumors developing between the 5th and 7th month, twenty-eight between the 7th and 9th, twelve between the 9th and 11th, and five between the 11th and 13th months. This is graphically illustrated in text-figure 1, and on this graph is superimposed a similar graph showing the incidence of tumors induced by *p*-dimethylaminoazobenzene (DAB), previously reported (*1*). The great similarity in the tumor incidence resulting from these two compounds is apparent from the crosshatched area of the overlap. No sex difference was found in morphology or incidence of the tumors.



TEXT-FIGURE 1.—Time of development of palpable liver tumors in Osborne-Mende rats fed *p*-dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N) superimposed on similar chart showing DAB-induced tumors (*1*). The same molal concentration of the respective azo dye was fed for the same length of time under similar conditions in these two experiments. The crosshatching shows areas of overlap and thus the essential similarity of the time distribution of the tumor incidence from these two compounds.

On gross examination, the earliest tumors appeared as discrete pinpoint lesions, clearly separated from the surrounding cirrhotic liver. On later examinations large areas of liver were seen to be replaced by nodular gray masses of tumor tissue (figs. 1, 3). The liver of every autopsied animal (except the one dying after only 18 days) had multiple small tumor nodules and most of the livers also had one or more large tumors. The largest tumor measured 7 × 5 × 5 cm. and weighed 67 gm. (about 20 percent of the body weight of the host). In 10 other rats there were massive tumors large enough to make up 10 to 20 percent of the body weight of the tumor-bearing animal. The cut surface of the small tumors was grayish white and firm. In larger tumors, necrosis, hemorrhage, and areas of liquefaction were common. The noncancerous liver tissue usually showed nodular portal cirrhosis and was often noted to have a yellow color. Small cysts were frequent. A bloody ascitic fluid was observed in one-third of the rats. In some instances it amounted to 30 to 40 cc. The icterus index of the blood was elevated to 15 to 30 units and the bilirubin content to 1 to 5 mg. per 100 ml. of serum in 20 percent of the animals. The rest of the animals had an icterus index of 4 to 5 units and no elevation in their serum bilirubin. The normal icterus index in Osborne-Mendel rats is one unit or less, with no measurable quantity of bilirubin. Bile staining of the organs was noted in many experimental animals.

Seventy percent of the animals had peritoneal spread of the tumor. This was massive and widespread in some rats (fig. 2). The omentum,

diaphragm, spleen, pancreas, stomach, kidneys, adrenal glands, and testes were involved. Pulmonary metastases (fig. 3) were found in 31 percent of the animals. However, most of these pulmonary metastases were noted on microscopic examination only. Bronchiectasis was frequent, as it often is in groups of old rats, but other organs usually appeared grossly normal.

Microscopically the livers showed a series of changes. The liver of the animal dying at 59 days showed diffuse necrosis in addition to small hepatomas. Portal cirrhosis, vacuolar changes in small scattered areas, intralobular bile-duct proliferation, bile-duct dilatation and cyst formation, cholangiofibrosis in scattered areas, foci of extramedullary hematopoiesis, and pigment deposits within macrophages were found in areas of the liver not involved by tumor in rats kept on the carcinogenic diet for 59 days or longer.

The liver tumors histologically resembled those previously described for DAN and DAB (*1*). The majority of the tumors examined microscopically were classified as hepatomas. The next largest group was cholangiohepatoma (fig. 4). Very few tumors were either pure cholangiocarcinoma (fig. 5) or completely undifferentiated. All very large tumors were cholangiohepatomas, or pure hepatomas. All types could usually be found in the same liver, and hepatoma was usually the most frequent. In a few large cholangiohepatomas there were areas showing spindle-cell transformation of the tumor cells (fig. 6).

Slight to moderate mucin formation was commonly seen in cells lining the tubules of cholangiocarcinoma. Extensive necrosis and hemorrhage were found in the large tumors. Cartilage and osteoid were occasionally seen in the stroma of a few large tumors. Small foci of hematopoiesis were commonly seen in both large and small neoplasms.

The peritoneal spread of the liver tumors appeared generally to be the result of direct implantation on serosal surfaces, but sometimes invasion of lymphatics and blood vessels was seen microscopically. Histologically the implants were frequently of the cholangiohepatoma type. An undifferentiated carcinoma pattern was often present in the tumor implants. Epithelial mucin was often seen in the tumor nodules.

Tumor emboli of the hepatoma type were found in the pulmonary vessels in many animals. In a few animals metastases replaced large areas of pulmonary tissue. In table 1, the emboli and metastases are grouped under the heading "Metastases to lungs."

A variety of changes were observed in the other organs examined histologically. Bronchiectasis or chronic bronchitis was found in nearly all animals. In most of these rats tiny microscopic foci were observed in the lungs in which the alveolar walls were lined by cuboidal cells with large, darkly staining nuclei. These lesions were interpreted as the end stage of a focal inflammatory process. Spermatogenesis was reduced in most of the testes examined. Hyperplasia of the hematopoietic tissue in the spleen was found regularly, and there was moderate hemosiderosis. Superficial ulceration of the glandular stomach was occasionally seen and

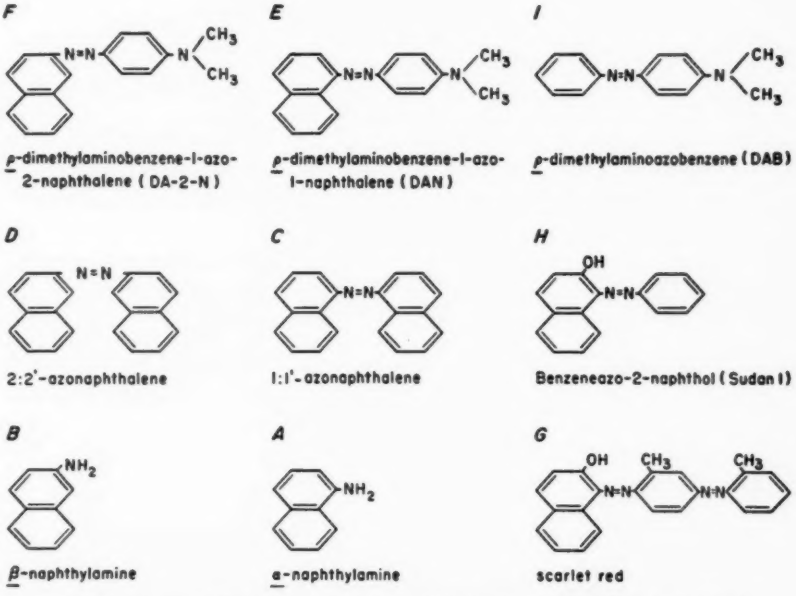
the squamous epithelium of the forestomach rarely showed focal atypical hyperplasia.

Histopathology of the rats painted with DA-2-N.—The 10 rats receiving the prolonged skin painting with 1-percent DA-2-N in benzene failed to develop either subcutaneous hemangioendothelioma, or tumor lesions in the liver as were observed in similarly treated mice in this laboratory (*3*). The livers in these rats were normal on gross and microscopic examination. At the site of the painting the epidermis showed atrophy, the hair follicles disappeared, and sebaceous glands were absent. These changes were probably related to the action of the benzene solvent.

Discussion

p-Dimethylaminobenzene-1-azo-2-naphthalene (text-fig. 2–F) induced various types of liver tumors with metastases to lungs and implantation metastases on many organs in the peritoneal cavity in Osborne-Mendel rats. It was interesting to compare the carcinogenic effects of this new carcinogen with that of similar compounds tried by other investigators to induce tumors in animals. Structural formulas of these compounds are shown in text-figure 2.

Fisher (*5*) in 1906 produced atypical epithelial proliferation of the skin with scarlet red (text-fig. 2–G), a dye containing an azonaphthol radical. Kirby and Peacock (*6*) induced liver tumors in mice with Sudan 1, a benzeneazo-2-naphthol (text-fig. 2–H). Cook *et al.* (*7*) produced more



TEXT-FIGURE 2.—Azo dyes and naphthylamines referred to in the text.

liver tumors in mice by feeding, painting, or injecting 2:2′-azonaphthalene (text-fig. 2–D) than with 1:1′-azonaphthalene (text-fig. 2–C). Liver tumors have not been reported in rats with any of these azonaphthalene-containing compounds. Sudan II, Yellow AB, and Yellow OB, all dyes similar to Sudan 1, did not produce tumors (*8*).

β-Naphthylamine (text-fig. 2–B) induced bladder tumors in rabbits (*9*), rats (*10*), dogs (*11*), and guinea pigs (*12*); while α-naphthylamine (text-fig. 2–A) did not (*8*). Therefore, it was of interest to compare the carcinogenic activity of DA-2-N (text-fig. 2–F), a β-azonaphthalene, to that of DAN (text-fig. 2–E), an α-azonaphthalene. As reported previously (*1*) the induction period [3] for DAN-induced tumors was 335 days, while that for DA-2-N–induced tumors was 150 days. Seventy-four percent of the DAN-induced tumors were found in 19 months, while 85 percent of the DA-2-N–induced tumors were found in only 11 months. As the concentration of DA-2-N in food (0.075 percent), the duration of its ingestion and the mean daily food intake per animal was the same as that for DAN, DA-2-N showed higher carcinogenic potency than DAN as judged by these criteria. This is in keeping with the reported higher carcinogenic activity of β-naphthylamine (text-fig. 2–B) and 2:2′-azonaphthalene (text-fig. 2–D) than that of α-compounds. Induction period for tumors induced by DAB (text-fig. 2–I) was 150 days and 85 percent of these tumors appeared in 11 months (text-fig. 1). DA-2-N was very similar to DAB in its induction period and the speed with which the tumors appeared.

The types of liver lesions produced with DA-2-N in rats and the frequency of each lesion are tabulated in table 1. They are compared with similar figures obtained for DAB and DAN in a previous study (*1*), carried out under identical conditions. DA-2-N induced hepatoma in at least 74 percent, cholangiohepatoma in at least 66 percent, cholangiocarcinoma in at least 12 percent, and undifferentiated tumor in at least 10 percent of the tumor-bearing rats. This azo dye produced lung metastases in 62 percent of the animals and tumor implants at 148 sites (organs) per 100 animals. DA-2-N behaves like DAN in its frequency of hepatoma and undifferentiated type of tumor, and like DAB in its frequency of mixed tumors. It differs from both DAN and DAB in producing large areas of cholangiocarcinoma, ascitic fluid, icterus of both tissues and serum, and a much greater frequency of intraperitoneal implants.

In this laboratory it was observed (*13*) that DAN induced liver tumors in about 75 percent of males but in only 18 percent of Osborne-Mendel females. Morris *et al.* (*14*) observed that male rats of strain A × C were considerably more susceptible than females to development of hepatoma by injection of 2-diacetylaminofluorene. Therefore, it was of interest to note that no sex difference was observed in induction of hepatomas with DA-2-N in this small series (16 females and 35 males) of Osborne-Mendel rats. All the females developed liver tumors. Time of incidence, induction period, size of tumors, and number of metastases and implants are

[3] Induction period is defined as the interval of time between the beginning of treatment with a carcinogen and the appearance of the first palpable tumor, in the group of animals so treated.

similar to those in the males. It is possible that administration of a lower concentration of the dye for a shorter time may bring out sex differences if any, in the susceptibility of the Osborne-Mendel rats to DA-2-N.

DA-2-N, a β-azonaphthalene compound, rarely induced atypical hyperplasia in the forestomach of the rat, though DAN, an α-azonaphthalene compound, had induced a series of forestomach changes including squamous-cell carcinoma (*2*). This failure to develop severe forestomach lesions with DA-2-N, may be due to earlier development of liver tumors with this compound. The changes observed in other organs of rats fed DA-2-N were not specifically determined to be due to the carcinogen. The occasional stomach lesions may have been related to the carcinogen. Splenic and testicular changes may be related indirectly to the treatment.

Osborne-Mendel rats painted with DA-2-N showed atrophy of the epidermis and subcutaneous glands, but neoplastic changes were not observed. In mice, however, skin painting with this compound produced hemangioendotheliomas at the site of painting (*3*).

Summary

p-Dimethylaminobenzene-1-azo-2-napthalene (DA-2-N), when fed to Osborne-Mendel rats, induced liver tumors in 100 percent of the 49 rats that lived 2 months or more after the start of the experiment. The first tumor induced by this azo compound was palpated about 150 days after the start of the experiment and 90 percent of the tumors appeared by 330 days.

This carcinogen induced hepatomas, cholangiocarcinomas, cholangiohepatomas, and undifferentiated tumors. Usually more than one type of tumor was present in one animal and often all types were found in the same animal. A study based on several liver sections from each animal revealed that at least 74 percent of the tumor-bearing animals had hepatomas, at least 66 percent had cholangiohepatoma, and at least about 10 percent each had cholangiocarcinoma and undifferentiated tumor. DA-2-N resembles DAN in its incidence of hepatoma and undifferentiated tumor. It resembles DAB in its shorter induction period and the incidence of cholangiohepatoma. Unlike DAN, DA-2-N failed to induce severe forestomach lesions.

References

(*1*) Mulay, A. S., and Firminger, H. I.: Liver tumors induced in rats by *p*-dimethylaminobenzene-1-azo-1-naphthalene compared with tumors induced by *p*-dimethylaminoazobenzene. J. Nat. Cancer Inst. 13: 35–55, 1952.

(*2*) ———: Precancerous and cancerous lesions of the forestomach and dermal subcutaneous tumors in rats fed *p*-dimethylaminobenzene-1-azo-1-naphthalene. J. Nat. Cancer Inst. 13: 57–71, 1952.

(*3*) Mulay, A. S., and Saxén, E. A.: Lesions induced in mice by painting with *p*-dimethylaminobenzene-1-azo-2-naphthalene. J. Nat. Cancer Inst. 13: 1259–1273, 1953.

(*4*) Kinosita, R.: The changes of the liver of the rat, caused by 4-dimethylaminobenzene-1-azo-1-naphthalene, 4-dimethylaminobenzene-1-azo-2-naphthalene, etc. Gann 34: 165–167, 1940.

(5) Fisher, B.: Die experimentelle Erzeugung atypischer Epithelwucherungen und die Entstehung bösartiger Geschwülste. Münch. med. Wschr. 53: 2041–2047, 1906.

(6) Kirby, A. H. M., and Peacock, P. R.: Liver tumours in mice injected with commercial food dyes. Glasgow M. J. 30: 364–372, 1949.

(7) Cook, J. W., Hewett, C. L., Kennaway, E. L., and Kennaway, N. M.: Effects produced in livers of mice by azonaphthalenes and related compounds. Am. J. Cancer 40: 62–77, 1940.

(8) Hartwell, J. H.: Survey of Compounds Which Have Been Tested for Carcinogenic Activity, 2d ed. Public Health Ser. Publ. No. 149. Bethesda, Md., National Cancer Institute, National Institutes of Health, 1951.

(9) Perlmann, S., and Staehler, W.: Zur Ätiologie der Blasengeschwülste. Ztschr. f. Urol. 27: 195–197, 1933.

(10) Yoshida, T., Shimauchi, T., and Kin, C.: Experimentelle Studien über die Entwicklung des Harnblasentumors. I. Mitt. Gann 35: 272–274, 1941.

(11) Hueper, W. C., Wiley, F. H., and Wolfe, H. D.: Experimental production of bladder tumors in dogs by administration of *beta*-naphthylamine. J. Indust. Hyg. & Toxicol. 20: 46–84, 1938.

(12) Satani, Y., Tanimura, T., Nishimura, I., and Isikawa, Y.: Klinische und experimentelle Untersuchung des Blasenpapilloma. Gann 35: 275–276, 1941.

(13) Mulay, A. S.: Unpublished data.

(14) Morris, H. P., Firminger, H. I., and Green, C. D.: Influence of sex hormones on development of hepatomas in A × C rats ingesting 2-diacetylaminofluorene. (Abstract.) Cancer Research 11: 270, 1951.

Plate 44

Typical tumors produced by feeding p-*dimethylaminobenzene-1-azo-2-naphthalene* (*DA-2-N*) *to Osborne-Mendel rats for 230 days*

Figure 1.—Large tumor replacing entire lobe of liver on the left side of the photograph. Many small tumor nodules in the remaining liver. × 1

Figure 2.—Multiple tumor nodules on the omentum in a rat which had large and small liver tumors. The large tumor has been removed. × 1

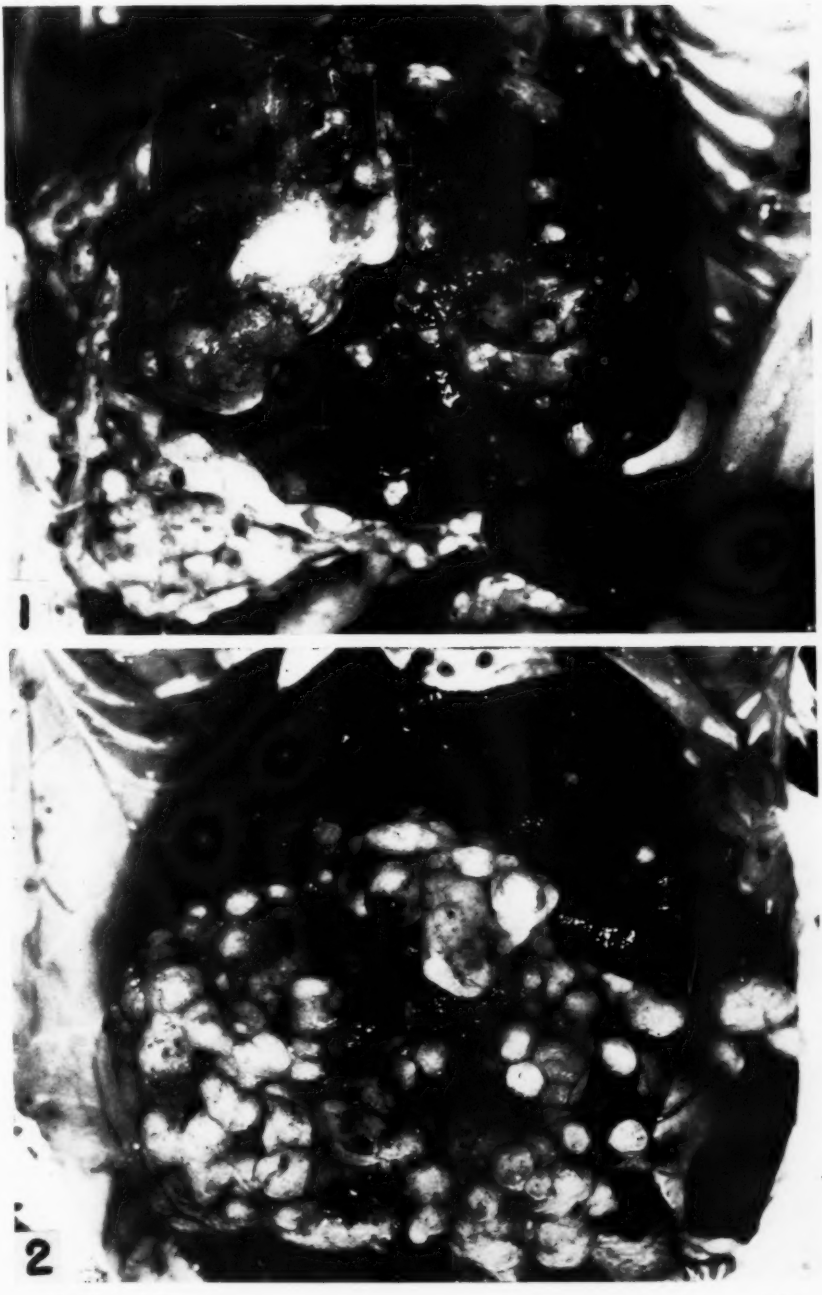

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PLATE 45

Typical tumors of the liver produced by feeding p-*dimethylaminobenzene-1-azo-2-naphthalene* (*DA-2-N*) *to Osborne-Mendel rats for 230 days.*

FIGURE 3.—Extensive pulmonary metastases from the large liver tumor seen in the right portion of the figure. × 1

FIGURE 4.—Cholangiohepatoma showing an area of cholangioma fairly sharply demarcated from surrounding hepatoma. Hematoxylin and eosin. × 165

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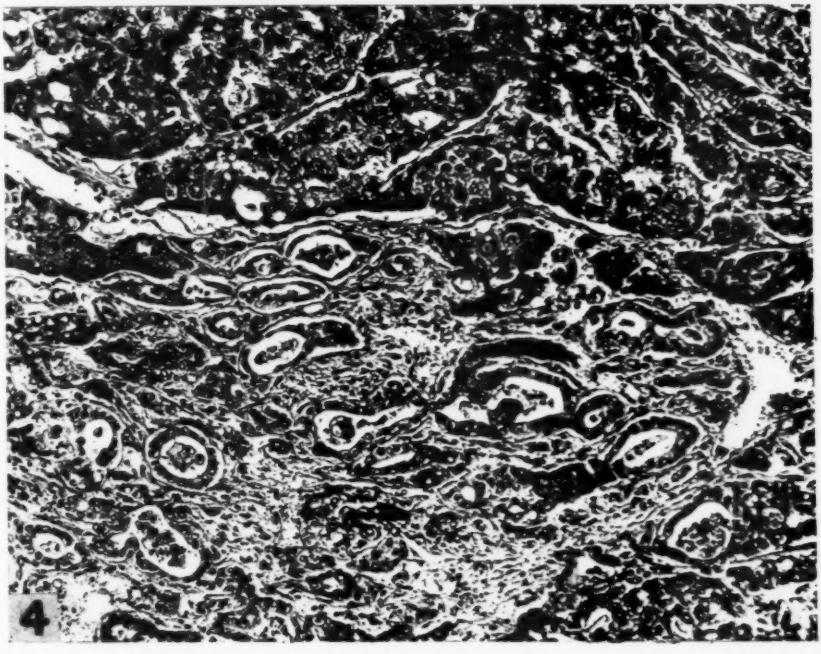
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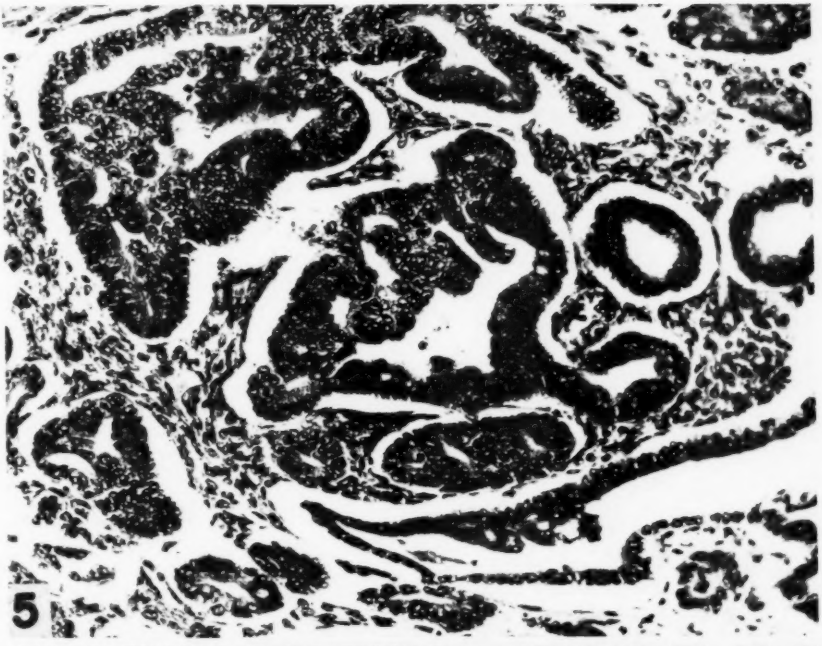
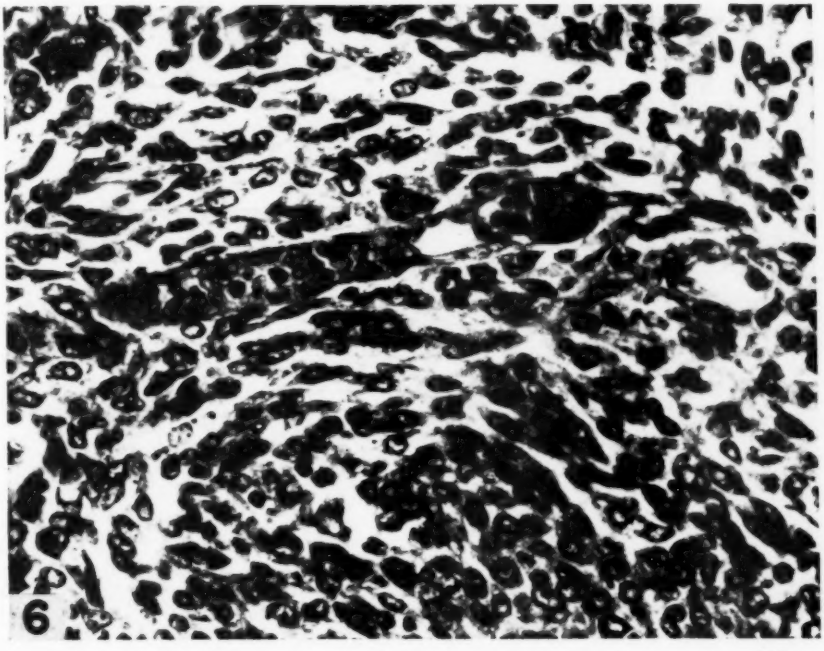
PLATE 46

Tumors of the liver produced by feeding p-*dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N) to Osborne-Mendel rats for 230 days. Sections stained with hematoxylin and eosin.*

FIGURE 5.—Cholangiocarcinoma of the liver. × 270

FIGURE 6.—Spindle-cell transformation of undifferentiated tumor cells around a small blood vessel. × 400





273201—53——14

Enzyme Changes Induced in Normal and Malignant Tissues with Chemical Agents. III. Effect of Acetylpodophyllotoxin-ω-Pyridinium Chloride on Cytochrome Oxidase, Cytochrome C, Succinoxidase, Succinic Dehydrogenase, and Respiration of Sarcoma 37 [1]

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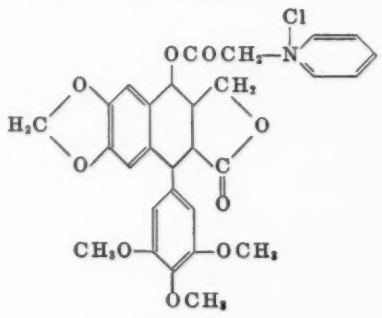
In two previous publications (*1, 2*) we demonstrated a marked drop in the cytochrome oxidase activity in homogenates of transplanted tumors occurring shortly after a single subcutaneous injection of a variety of compounds capable of inducing damage in these tumors. Much smaller changes in enzyme content were observed in normal organs from both tumor-bearing and non-tumor-bearing mice. The present report furnishes additional data on the influence of these compounds on related components of the cytochrome system other than cytochrome oxidase.

Materials and Methods

The compound employed in these experiments was a soluble derivative of podophyllotoxin, *acetylpodophyllotoxin-ω-pyridinium* chloride (NCI-3022), prepared by Schrecker and Hartwell [4] by quaternizing a derivative of acetylpodophyllotoxin. This compound was found to be very soluble in water and to be about as potent as podophyllotoxin in producing damage in transplanted tumors.[5] Its high water-solubility made this compound more convenient to use than the parent substance podophyllotoxin.



CAF_1 mice, some bearing six-day-old intramuscular implants of Sarcoma 37, were employed in all experiments. The mice received a single sub-

[1] Received for publication July 22, 1953.
[2] Visiting Scientist, National Institutes of Health, Bethesda, Md.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[4] Unpublished data.
[5] Details of the effects of this compound on Sarcoma 37 will be published in a separate report.

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cutaneous injection of the compound (NCI-3022) and were sacrificed in groups of at least 4 animals each, at intervals from 2 to 48 hours after injection. Tissues from about four animals were pooled, divided into two parts, and homogenates of the tumor and normal tissues were prepared as previously described (*1*).

Cytochrome oxidase activity was determined according to the method of Schneider and Potter (*3*) in 1 percent tissue homogenates; autoxidation was determined by extrapolation to zero tissue concentration. Succinoxidase activity was determined in 5 percent tissue homogenates following the method of Schneider and Potter (*3*), and succinic dehydrogenase activity was measured according to the method of Slater (*4*). Succinic dehydrogenase activity was also determined following the procedure of Quastel and Wheatley (*5*) using sodium succinate and potassium ferricyanide, the latter replacing methylene blue. Similar values were obtained by both methods. The amount of tissue used depended on the enzyme activity. Respiration of tissue slices was determined by a manometric procedure in Krebs-Ringer-phosphate buffer at 37.5° C. in an atmosphere of oxygen. The tissue slices were approximately one millimeter thick and weighed about 50 milligrams. After determining the respiration, the slices were washed with distilled water, dried, and weighed.

The procedure for extraction of cytochrome c from tissue, removal of hemoglobin, precipitation and spectrophotometric determination was similar to that of Rosenthal and Drabkin (*6*). In the case of liver, the homogenate was incubated for 30 minutes with a solution of filtered saliva to hydrolyze glycogen. The extraction procedure for this tissue was then carried out in the same way as for the other tissues.

Results

Table 1 gives the results of a single subcutaneous injection of 20 μg. per gm.[6] of NCI-3022 on the cytochrome oxidase and cytochrome c content of homogenates of Sarcoma 37 and of liver. The pattern of reduction in cytochrome oxidase activity was similar to that reported previously (*1*) for its parent compound, podophyllotoxin (NCI-1073).[7] Analogously, the level of endogenous cytochrome c in the tumor tissue decreased in the same way as the cytochrome oxidase. The cytochrome c content of liver tissue was scarcely affected.

The effect of injection of a similar dose of NCI-3022 on succinoxidase activity of homogenates and on the respiration of slices of tumor and liver is shown in table 2. The decreases in the activity of this enzyme and in the respiration were more precipitous and pronounced than in the cytochrome components. In particular, only about 40 percent of the succinoxidase activity of untreated controls remained in the tumors after as

[6] This dose is approximately four times the minimum effective dose (MED) and about one-fourth the maximum tolerated dose (MTD).

[7] In unpublished experiments of Kelly and Leiter, in this Laboratory, and in some of the *in vitro* experiments given later in this paper, there appeared to be some evidence that NCI-3022 undergoes hydrolysis in the presence of tissues to yield podophyllotoxin. It is quite likely that the action of this compound may be due to the liberation of the parent compound. More recently Schrecker has isolated podophyllotoxin from aqueous solutions of the compound buffered at *p*H 7.3.

TABLE 1.—*Effect of a single injection of 20 μg. per gm. of NCI-3022 on the cytochrome oxidase activity and cytochrome c content of Sarcoma 37 and liver homogenates*

Time after injection (*hours*)	Residual activity*†			
	Cytochrome oxidase		Cytochrome c	
	Tumor (*percent*)	Liver (*percent*)	Tumor (*percent*)	Liver (*percent*)
2	89	100	89	101
4	78	100	79	94
24	66	102	59	92
48	42	100	42	90

*$\frac{\text{Activity of treated}}{\text{Activity of control}} \times 100$.

†Control values: *cytochrome oxidase* (Q_{O_2} in cu. mm. of O_2 per mg. dry tissue per hr.)

Sarcoma 37. . . . 127–136
Liver 318–328
cytochrome c (μg. per gm. of dry tissue)
Sarcoma 37. . . . 224–241
Liver 396–413

short a period as 4 hours following injection of the compound. By 24 hours, the enzyme activity was scarcely measurable. Again, only a negligible effect was observed with liver homogenates or slices.

Text-figure 1 gives the results obtained with different dose levels of NCI-3022 on succinoxidase activity in Sarcoma 37. A dose of 2 or 4 μg. per gm. appeared to have little effect. Appreciable decreases in the enzyme activity appeared only after 24 hours following a dose of 8 μg.

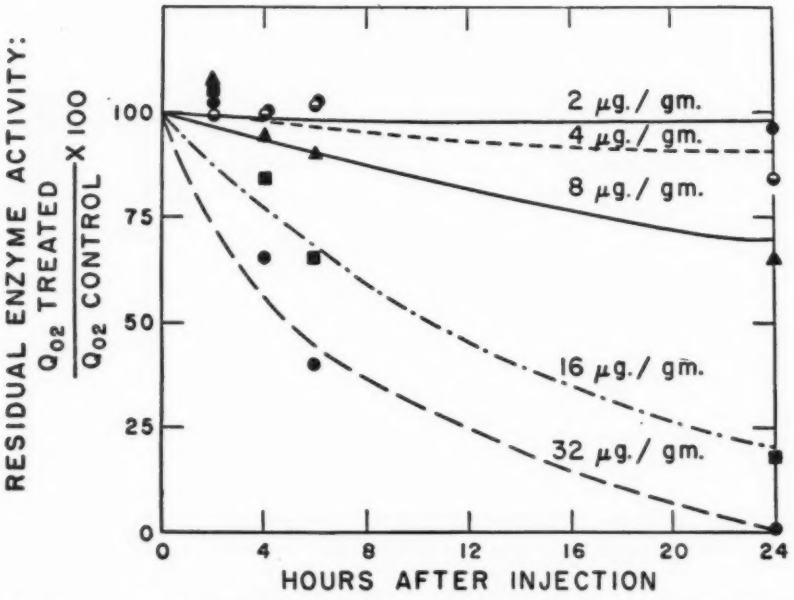
TABLE 2.—*Effect of a single injection of 20 μg. per gm. of NCI-3022 on succinoxidase and respiration of Sarcoma 37 and of liver*

Time after injection (*hours*)	Residual activity*†			
	Succinoxidase (*homogenates*)		Respiration (*slices*)	
	Tumor (*percent*)	Liver (*percent*)	Tumor (*percent*)	Liver (*percent*)
2	96	104	72	103
4	40	109	40	91
24	2	94	38	92
48	10	104	31	102

*$\frac{\text{Activity of treated}}{\text{Activity of control}} \times 100$.

†Control values: Q_{O_2} (cu. mm. of O_2 per mg. dry tissue per hr.)

succinoxidase
Tumor. 17.7–22.7
Liver. 91–103
respiration
Tumor. 7.0–8.2
Liver. 7.6–8.4



TEXT-FIGURE 1.—Effect of different dose levels of NCI-3022 on succinoxidase activity of Sarcoma 37.

per gm. The minimum effective dose of NCI-3022 against Sarcoma 37 is approximately the same (5 μg. per gm.). The highest dose given in this series (32 μg. per gm.), which produced a 60 percent decrease in enzyme activity in 6 hours, was about one-third of the maximum tolerated dose (80 μg. per gm.).

Injection of a maximum tolerated dose (80 μg. per gm.) of NCI-3022 produced comparatively slight changes in the succinoxidase activity of liver, kidney, spleen, and lung of non-tumor-bearing mice. The results are summarized in table 3. It should be noted that the enzyme activity of spleen and lung were in the same range as the activity in Sarcoma 37 from untreated animals (table 2).

In contrast to the effects on the succinoxidase system, succinic dehydrogenase activity of tumor homogenates was scarcely affected by a dose of 20 μg. per gm. of NCI-3022. Following even a lethal dose of 100 μg. per gm., about 80 percent of the enzyme activity remained for as long as 48 hours after injection, at a time when necrosis was quite extensive in the tumors. These results are shown in text-figure 2.

In vitro exposure of slices of Sarcoma 37 to a concentration of 100 or 250 μg. per ml. of NCI-3022 or of podophyllotoxin (NCI-1073) for 4 hours, produced smaller decreases in succinoxidase activity than were observed in the *in vivo* experiments. Table 4 summarizes the results. Even when tumor homogenate was employed, the *in vitro* addition of the compound (NCI-3022) in concentrations up to 500 μg. per ml. failed to inhibit the suc-

TABLE 3.—*Effect of a single injection of 80 μg. per gm. of NCI-3022 on succinoxidase activity of tissues from non-tumor-bearing CAF_1 mice*

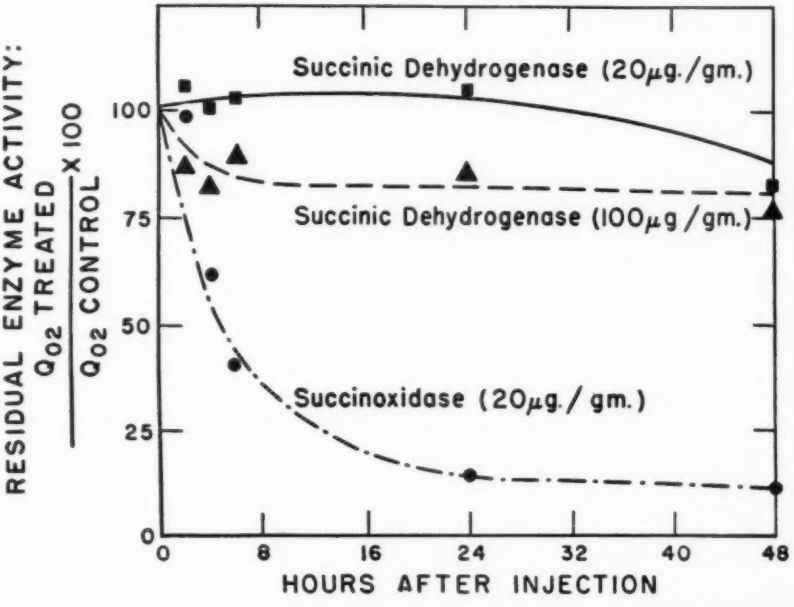
Time after injection (*hours*)	Residual activity*†			
	Liver (*percent*)	Kidney (*percent*)	Spleen (*percent*)	Lung (*percent*)
2	104	92	104	103
4	98	96	103	102
6	102	80	99	87
24	—	80	78	84
48	90	90	79	92

* $\frac{\text{Activity treated}}{\text{Activity control}} \times 100$.

† Control values: Q_{O_2} (cu. mm. of O_2 per mg. dry tissue per hr.)

Liver 117–120 Spleen 20.3–22.4
Kidney 210–247 Lung 15.6–18.5

cinoxidase activity of the tumor markedly. Similar concentrations of colchicine (NCI-1v), carried out at the same time, produced even smaller changes. Table 5 shows that the effects produced *in vitro* on respiration of slices were similar to those for succinoxidase.



TEXT-FIGURE 2.—Effect of NCI-3022 on succinic dehydrogenase and succinoxidase activity of Sarcoma 37. Control Q_{O_2} (cu. mm. O_2 per mg. dry tissue per hour) for succinic dehydrogenase was 6.0–7.0, and for succinoxidase 19.2–20.6.

TABLE 4.—*Succinoxidase activity of tumor slices treated with NCI-3022, podophyllotoxin, or colchicine* in vitro

Time of incubation	Residual activity*†					
	NCI-3022		Podophyllotoxin		Colchicine	
(*hours*)	100 μg./ml. (*percent*)	250 μg./ml. (*percent*)	100 μg./ml. (*percent*)	250 μg./ml. (*percent*)	250 μg./ml. (*percent*)	100 μg./ml. (*percent*)
1	87	100	87	80	85	86
2	73	80	66	64	87	76
3	73	75	75	64	104	90
4	84	78	78	75	97	94

*The values shown are percentages of the residual enzyme activity of tumor slices in contact with the compound.

†Q_{O_2} (cu. mm. O_2 per hr. per mg. dry tissue) of tumor slices before addition of compound was 6.3–8.3. The loss in enzyme activity of tumor slices in the absence of compound during five hours of incubation was about 8 percent.

TABLE 5.—*Respiration of tumor slices treated with NCI-3022, podophyllotoxin, or colchicine* in vitro

Time of incubation	Residual activity*							
	NCI-3022			Podophyllotoxin			Colchicine	
(*hours*)	20 μg./ml. (*percent*)	100 μg./ml. (*percent*)	250 μg./ml. (*percent*)	20 μg./ml. (*percent*)	100 μg./ml. (*percent*)	200 μg./ml. (*percent*)	20 μg./ml. (*percent*)	100 μg./ml. (*percent*)
1	104	97	72	107	101	71	111	106
2	76	78	73	85	77	63	84	85
3	72	69	61	82	60	54	92	87
4	67	60	58	79	58	52	82	81

*The values shown are percentages of the residual respiration of tumor slices after the addition of the compound. The respiration of tumor slices before addition of compound was Q_{O_2} (cu. mm. O_2 per hr. per mg. dry tissue) 7.5–8.5. The loss in respiration of tumor slices in the absence of compound during five hours of incubation was about 6 percent.

Discussion

The data presented here show that the enzymatic effects that followed injection of tumor-damaging compounds were not restricted to cytochrome oxidase; that cytochrome c, succinoxidase, and over-all respiration were equally or more markedly affected, whereas succinic dehydrogenase was least affected. Furthermore, the reduction in succinoxidase cannot be attributed to the cytochrome system as a limiting factor. As in the case of the cytochrome oxidase determination, cytochrome c was added to the substrate. Cytochrome oxidase was not a limiting factor either, since the concentration of this enzyme, present at any time interval after injection of compounds, was greater than that of succinoxidase even in untreated tissue. Finally, the decrease in succinoxidase was so much more marked than that of cytochrome oxidase, that virtually no succin-

oxidase could be demonstrated after 24 hours. It appears, therefore, that the succinoxidase system is even more susceptible to effects produced by tumor-damaging compounds than cytochrome oxidase.

It should be noted that the Q_{O_2} values for succinic dehydrogenase activity are about one-third of the values observed for the succinoxidase system, indicating that the methods used (*4*, *5*) did not measure the entire activity of this enzyme. They are, however, comparable to the values reported by Slater (*4*) and by Potter and Reif (*7*), who also obtained dehydrogenase activities of only one-third that of the succinoxidase activity. Despite these lower values, the dehydrogenase activity remained relatively constant throughout the experiments, whereas the succinoxidase activity dropped precipitously. In fact, at time intervals longer than 8 hours after injection of the compound, the absolute values for succinic dehydrogenase were *greater* than those for succinoxidase. It is apparent then, that a factor other than the dehydrogenase was the limiting feature in the loss of succinoxidase activity.

One factor which might conceivably play a role in the marked effect on the succinoxidase system with little or no effect on the succinic dehydrogenase activity is that suggested by Slater (*8*) in his work with BAL. This factor was postulated to be a component of the succinoxidase system required for the transmission of electrons between cytochrome b (succinic dehydrogenase) and cytochrome c. Whether this is a consequence of a specific effect, such as described by Slater for BAL, or to "nonspecific" effects such as are ascribed to certain toxins (*9*), remains to be established.

The reduction in respiration of tissue slices treated *in vivo* with NCI-3022 is in accord with the reports of Boyland and Boyland (*10*) and of Miller *et al.* (*11*) on the decrease in respiration of tumor slices following injection of colchicine or of podophyllotoxin.

It does not appear that these effects are related to the type of "enzyme titration" postulated by Ackermann and Potter (*12*), as exemplified by the effect of antimycin A on succinoxidase activity (*7*). Of 5 tissues examined, only the enzyme activity of tumor was markedly affected. Even though both spleen and lung had a low succinoxidase activity, comparable with that of Sarcoma 37, the activity in these tissues was not lowered percentage-wise to a greater extent than in tissues of much higher enzyme activity (liver and kidney, see table 3). Nevertheless spleen is affected grossly and microscopically by podophyllotoxin (*13*). Conceivably, factors such as blood circulation could play a role in this situation, as was postulated by Reif and Potter (*14*) in the case of discrepancies between *in vivo* and *in vitro* effects of antimycin A.

Work is in progress on the effects of tumor-damaging compounds on other enzymes participating in carbohydrate metabolism, as well as on enzymes unrelated to this metabolic process.

Summary

1) A single subcutaneous injection of 20 μg. per gm. of *acetylpodophyllotoxin-ω-pyridinium chloride*, in mice bearing Sarcoma 37, produced a

marked drop in cytochrome oxidase activity, cytochrome c and succinoxidase activity of tumor tissue homogenates. A marked decrease in respiration of tumor tissue slices was also observed. However, only a small reduction in succinic dehydrogenase activity was seen even with a lethal dose of the compound.

2) In liver, kidney, spleen, and lung of tumor-bearing mice treated with a maximum tolerated dose (80 μg. per gm.) of this compound, a much smaller percentage decrease in succinoxidase activity was observed than in tumor tissue.

3) *In vitro* incubation of tumor slices with *acetylpodophyllotoxin-ω-pyridinium chloride* (NCI-3022), podophyllotoxin, or colchicine in concentrations up to 250 μg. per ml. produced smaller changes in succinoxidase activity and respiration than those observed after the same interval following treatment *in vivo*.

References

(*1*) WARAVDEKAR, V. S., DOMINGUE, A., and LEITER, J.: Effect of podophyllotoxin, *alpha*-peltatin and *beta*-peltatin on the cytochrome oxidase activity of Sarcoma 37. J. Nat. Cancer Inst. 13: 393–407, 1952.

(*2*) LEITER, J., PARADIS, A. D., and WARAVDEKAR, V. S.: Enzyme changes induced in normal and malignant tissues with chemical agents. II. Effect of various compounds on the cytochrome oxidase activity of transplanted tumors. J. Nat. Cancer Inst. 14: 177–188, 1953.

(*3*) SCHNEIDER, W. C., and POTTER, V. R.: The assay of animal tissues for respiratory enzymes II. Succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem. 149: 217–227, 1943.

(*4*) SLATER, E. C.: A comparative study of the succinic dehydrogenase-cytochrome system in heart muscle and in kidney. Biochem. J. 45: 1–8, 1949.

(*5*) QUASTEL, J. H., and WHEATLEY, A. H. M.: CXXVI Anaerobic oxidations. On ferricyanide as a reagent for the manometric investigation of dehydrogenase systems. Biochem. J. 32: 936–943, 1938.

(*6*) ROSENTHAL, O., and DRABKIN, D. L.: Spectrophotometric studies. XI. The direct micro-spectrophotometric determination of cytochrome c. J. Biol. Chem. 149: 437–450, 1943.

(*7*) POTTER, V. R., and REIF, A. E.: Inhibition of an electron transport component by antimycin A. J. Biol. Chem. 194: 287–297, 1952.

(*8*) SLATER, E. C.: A respiratory catalyst required for the reduction of cytochrome c by cytochrome b. Biochem. J. 45: 14–30, 1949.

(*9*) ———: The action of inhibitors on the system of enzymes which catalyse the aerobic oxidation of succinate. Biochem. J. 45: 8–13, 1949.

(*10*) BOYLAND, E., and BOYLAND, M. E.: Studies in tissue metabolism. IX. The action of colchicine and *B. typhosus* extract. Biochem. J. 31: 454–460, 1937.

(*11*) MILLER, Z. B., DAVISON, C., and SMITH, P. K.: The effect of podophyllotoxin on tissue metabolism and enzyme systems. J. Exper. Med. 90: 525–541, 1949.

(*12*) ACKERMANN, W. W., and POTTER, V. R.: Enzyme inhibition in relation to chemotherapy. Proc. Soc. Exper. Biol. & Med. 72: 1–9, 1949.

(*13*) GREENSPAN, E. M., LEITER, J., and SHEAR, M. J.: Effect of *alpha*-peltatin, *beta*-peltatin and podophyllotoxin on lymphomas and other transplanted tumors. J. Nat. Cancer Inst. 10: 1295–1333, 1950.

(*14*) REIF, A. E., and POTTER, V. R.: *In vivo* inhibition of succinoxidase activity in normal and tumor tissues by antimycin A. Cancer Research 13: 49–57, 1953.

Radioiodide-Concentrating Ability of Transplantable Tumors of the Thyroid Gland in C3H Mice [1]

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Thyroid tissue accumulates I^{131} after radioiodide is injected into animals. Almost all of the accumulated I^{131} is bound in organic combination. The remainder of the accumulated I^{131}, radioiodide, although making up only a small fraction of the thyroid I^{131}, is maintained at a concentration many times the radioiodide concentration in the serum. The ratio of the radioiodide concentration in tissue to that in serum (T/S ratio) is a measure of the radioiodide-concentrating ability of the tissue. If the T/S ratio for the tissue is greater than 1, the tissue is said to have radioiodide-concentrating ability. Transplantable tumors of the thyroid gland in C3H mice (*1*, *2*) also accumulate I^{131}, but the amount per gram of tissue is significantly less than that in the thyroid gland in the same animal (*3*).

In the present experiments the radioiodide-concentrating ability of several lines of transplantable tumors of the thyroid gland was measured. It was found that the radioiodide-concentrating ability of tumors of all lines was less than that of thyroid gland; in independent [3] tumors, radioiodide-concentrating ability was almost completely absent. In some tumor lines the inability to concentrate radioiodide appeared to play an important part in their loss of ability to accumulate I^{131}.

Materials and Methods

Tumors of lines 4H, 4J, 3A, 4, 3C, and 6 were studied. Lines 4H and 4J which appear to be indistinguishable by histologic and radioiodine-uptake criteria have been designated earlier as two of the components of dependent line 4HJK (*3*). Certain characteristics of tumor line 4HJK and of independent lines 3C and 6 have been described by Wollman, Scow, Wagner, and Morris (*3*), but lines 3A and 4 have not been described before. Up to the generation used in this study (Generation 9), tumors of line 3A have been dependent. However, some tumors of this generation and also transplanted pieces of at least one tumor of this generation

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[3] These tumors are called "dependent" or "independent" according to whether or not their growth depends upon their hosts being fed thiouracil.

grew in hosts fed a thiouracil-free diet, and therefore were independent (table 1).

Tumor inoculation.—Tumor inoculations were made from one or more tumors of a selected line into either the subcutaneous flank, thigh muscle, or both, in each of a group of young male mice. The inoculation sites were the same for a given group.

Diet.—After inoculation each group of mice was fed either a pelleted stock diet, or a meal diet of similar composition but containing in addition 2.5 grams of thiouracil for every kilogram of diet (*3*), (table 1). For a time interval of from 7 to 21 days immediately prior to the radioiodine studies the mice, except for those of one group, were fed a moderately low-iodine diet (*3*) as indicated in table 1. Two separate batches of the low-iodine diet were used. Although it was intended that these batches be duplicates, they appeared to differ in their iodine content. Results obtained in mice fed diet batch *a* were generally higher than those in mice fed batch *b*.

TABLE 1.—*Experimental details*

Tumor line	Generation studied	Site* of tumor inoculations	Number of days fed diet after tumor inoculations		
			Thiouracil	Stock	Low iodine†
4H, 4J	11	SC	120 to 140	.....	0 to 21
4H	12	SC	102	.....	7
3A	9	SC+IM	129	.....	7
4	11	SC	.....	140	7
3C	11	SC+IM	.....	111	14
6	11	SC+IM	.....	96	14

*SC=subcutaneous; IM=intramuscular.
†Immediately preceding I^{131} studies.

Measurements of the T/S ratio when binding was blocked and when binding was permitted were made at the same time in duplicate groups of mice bearing tumors of a given line. An attempt was made to have as wide a weight range of tumors as possible in each duplicate group. Procedures used were similar to those described by Wollman and Scow (*4*).

Blocking of organic binding.—Organic binding of radioiodide was prevented by a subcutaneous injection of 2.5 mg. of propylthiouracil dissolved in 0.5 ml. of a 0.9 percent NaCl solution which was 0.04 N in NaOH. This was administered approximately one hour prior to radioiodide injection. This quantity of propylthiouracil has been shown by Wollman and Scow (*4*) to block binding of radioiodide in the thyroid glands of mice. Blocking of binding in tumors was tested by homogenizing tumors in 10 ml. of 8 percent trichloroacetic acid (TCA) and comparing the amounts of non-precipitable and of precipitable I^{131} with total tumor I^{131}.

I^{131} injection.—Ten microcuries of carrier-free radioiodide [4] (I^{131}) in one milliliter of water were injected intraperitoneally into each mouse.

[4] The I^{131} was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn.

Procedure at autopsy.—Between one and three hours after the radioiodide injection, each mouse was anesthetized with ether and a blood sample was obtained from the heart. The thyroid gland and tumor were then immediately removed. Tumors were examined grossly for necrosis immediately after excision. Necrotic material, rarely encountered, was discarded. The thyroid gland and tumor were then weighed and placed individually into either a counting vessel containing Zenker's formol or into a glass homogenizer containing 10 ml. of ice-cold 8-percent TCA and 1 mg. NaI.

The I^{131} in the supernatant after homogenization and centrifugation was termed nonprecipitable I^{131} and was assumed to be radioiodide. Preliminary experiments had shown that if I^{131} was present in thyroid homogenates only in the form of radioiodide, 98.5 percent of the I^{131} was found in the supernatant provided that carrier iodide was added. If no carrier was added only 85 percent of the I^{131} was found in the supernatant. However, if protein-bound I^{131} is present in the tissue it has been shown by Taurog, Tong, and Chaikoff (*5*) that the deproteinization of thyroid homogenates by TCA is not complete. The supernatant contains organic bound I^{131} largely in the form of protein. This makes the estimates of the radioiodide content of the tissue high. In the case of the tumors, however, the error appears to be small since the nonprecipitated protein was only a small percent of the total protein and the amount of precipitable I^{131} was usually not more than six times that of the nonprecipitable I^{131}. In this case if only 97 to 98 percent of the iodine-containing organic matter were precipitated [as appears to be the case for thyroid gland (*4*)] the estimate of radioiodide would be only 12 to 18 percent high.

The serum I^{131} concentration was used in place of the serum radioiodide concentration, since it was found that the percent of the serum I^{131} which was protein bound was negligible for the time interval after injection during which the present studies were made.

I^{131} Measurements.—The I^{131} content in the tissues or in fractions obtained from tissues was determined by gamma counting using the well-type counter described earlier (*6*). The total amount of I^{131} in serum was determined by counting beta radiation from weighed aliquots of serum or of diluted serum as described by Wollman and Scow (*4*). Self-absorption corrections were made where necessary. The statistical counting error was less than 5 percent by either method. The counting rate of a sample using the beta counter was 150 times that using the gamma counter. When computing T/S ratios, data on thyroid gland or its fractions were converted to values that would have been obtained if beta counting had been used.

Results

Histology.—At least one histologic section was obtained from each tumor that was not homogenized. Sections stained with hematoxylin and eosin were examined for microscopic necrosis. Only a few tumors showed histologic evidence of necrosis, and then only a negligible fraction of any tissue section was involved.

Microphotographs of typical sections of tumors of line 3A and of line 4 are shown in figures 1, 2, and 3. Microphotographs of sections of two tumors of line 3A are shown because the tumors of this line were of two different types, one more like line 4HJK, the other resembling lines 3C or 6.

Nonprecipitable I^{131} in thyroid glands and tumors of the thyroid gland.—When binding was permitted, 6 to 9 percent of the I^{131} in hyperplastic thyroid glands was nonprecipitable approximately two hours after radioiodide injection (table 2). A greater proportion of the I^{131} was nonprecipitable in tumors than in the gland. However, nonprecipitable I^{131} in a tumor was never over 30 percent of the total tumor I^{131}.[5]

TABLE 2.—*Percent of tissue I^{131} that was nonprecipitable with TCA**

Tumor line	Organic binding blocked		Organic binding permitted	
	Thyroid	Tumor	Thyroid	Tumor
4H (Gen. 11)	.....	82 (1)†	.....	1.33 (1)
4H (Gen. 12)	92.8 (2)	92.8 (2)	5.6 ± 0.6‡(8)	18.4 ± 3.4 (8)
3A	98.3 (2)	88.3 (2)	9.1 ± 2.2 (3)	17.5 ± 5. (5)
4	.....	93 (1)	.....	20.6 (1)
3C	.....	84.5 (2)	.....	12.8 ± 4 (4)
6	.....	89.7 (2)	.....	14.8 ± 5 (4)

*Approximately 2 hours after radioiodide injection.
†Number of samples used in determination of average.
‡Standard error of the mean.

After blocking organic binding of I^{131}, the nonprecipitable I^{131} in thyroid glands was found to be over 90 percent of the total I^{131} (table 2). In tumors, approximately 90 percent of the total was generally nonprecipitable. This value for tumors must be considered to be a low estimate because the I^{131} in the supernatant was measured after only a single homogenization and sedimentation, and the sediment was not washed and therefore contained supernatant and hence nonprecipitable I^{131}. The tumors of line 6 made up over 5 percent of the volume of the homogenate and those of line 3C more than 10 percent. In the latter case, if I^{131} were distributed uniformly between the supernate and the sediment, the I^{131} in the supernate could not be expected to exceed 95 percent of the total tumor I^{131}. Under these conditions less than 10 percent of the total I^{131} in the tumors can be considered to be precipitable.

T/S ratio when binding was blocked.—When binding was blocked, thyroid glands in tumor-bearing animals had T/S ratios averaging from 90 to 250, the magnitude depending on whether batch *b* or *a* of the low-iodine diet was fed and whether the host had been fed a thiouracil-containing diet (table 3). The T/S ratio obtained for the tumors was always significantly lower than that for the thyroid gland in the same

[5] This result excludes the possibility discussed elsewhere (7) that the lack of constancy of the ratio of the uptake per mg. of tumor to that of thyroid gland observed for line 6 (5) might be due to radioiodide making up a very large fraction of the total tumor I^{131}.

TABLE 3.—*The ratio of tissue radioiodide concentration to serum radioiodide concentration (T/S)*

Tumor line	Growth dependence	Diet	Days fed low-iodine stock diet*	Organic binding blocked			Organic binding permitted			
				Thyroid	Tumor		Thyroid	Tumor		
				T/S	T/S	Weight range (*mg.*)	T/S	T/S	Weight range (*mg.*)	$\frac{\text{Total } I^{131} \text{ per mg. tumor} \times 10^3}{\text{Total } I^{131} \text{ per mg. thyroid}}$
4J	Dependent	Thiouracil	7 (*a*)	256 ± 16† (6)‡	61. ± 6 (6)	267–842				
4H	Dependent	Thiouracil	7 (*b*)	91 ± 10 (6)	33. ± 3 (6)	224–305	15.2 ± 0.6 (8)	12.8 ± 1.3 (8)	65–362	296. ± 24. (8)
3A	In transition	Thiouracil	12 (*b*)	110 ± 23 (7)	9.2 ± 3 (6) 1.01 ± 0.5 (5)	29–101 233–563	20 ± 4 (3)	1.8 ± 0.5 (5)	120–393	53.2 ± 15. (9)
4	Independent	Stock	7 (*a*)	171 ± 60 (3)	2.3 ± 0.7 (3)	156–534				49. ± 38. (3)
3C	Independent	Stock	14 (*b*)	120 ± 19 (7)	0.42 ± 0.02 (7)	163–1350		0.37 ± 0.04 (4)	330–1160	4.5 ± 1.3 (8)
6	Independent	Stock	14 (*b*)	149 ± 26 (6)	0.39 ± 0.01 (12)	814–3400		0.35 ± 0.02 (4)	442–1270	7.1 ± 1.4 (7)

*See text for discussion of diet batches *a* and *b*.
†Standard error of the mean.
‡Number of determinations.

animal. The T/S ratio in tumors of the dependent line 4H averaged 33 (never over one-half that of the thyroid); that in tumors of independent lines averaged less than 2 (table 3).

The value of the T/S ratios for tumors of lines 4H, 3C, and 6 appear to be independent of tumor weight over the weight range indicated in table 3. There were too few tumors of line 4 to allow demonstration of any weight dependence. In tumors of line 3A, however, a definite dependence of the T/S ratio on tumor weight was observed. When organic binding was blocked, the T/S ratio in six tumors of this line weighing less than 100 mg. averaged 9.2, whereas that in five tumors weighing over 233 mg. averaged 1.01. This observation may be related to the fact that these two groups of tumors differed histologically from each other, and independent tumor tissue was first detected in the present generation (Generation 9) of this tumor line. The smaller tumors of line 3A resembled tumors of line 4HJK both histologically and biochemically, whereas the larger tumors resembled those of line 3C (figs. 1 and 2).

T/S ratio when binding was permitted.—When binding was permitted the T/S ratio for hyperplastic thyroids was found to be approximately 15 to 20 (table 3). The T/S ratio for tumors of the dependent line 4H was approximately equal to the T/S ratio for the thyroid gland in the same host. In contrast, tumors of all independent lines had T/S ratios averaging less than 2.

Effect of the tumor on the thyroid T/S ratio.—Mice bearing tumors of dependent line 4H and tumor-free mice were fed the thiouracil diet for three months immediately preceding the I^{131} studies. The presence of the tumor was found to have no significant effect upon the thyroid T/S ratio (table 4).

Attempts to Alter the T/S Ratio of Tumors

1) *Effect of number of days fed stock diet after chronic feeding of thiouracil diet.*—A group of mice bearing tumors of dependent line 4J were fed the thiouracil diet for 130 days. They were then divided into two groups: one fed the low-iodine diet for 7 days, the other for 21 days. The T/S ratio for the thyroid gland was significantly higher in the mice fed the low-iodine diet for the shorter time interval, whereas the T/S ratio for the tumors was not significantly different in the two groups (table 4).

2) *Effect of hypophysectomy.*—A group of mice bearing tumors of dependent line 4H (Generation 11) were fed the thiouracil diet for 130 days. They were then fed the low-iodine diet for 7 days after which they were divided into two groups. One group was completely hypophysectomized, the other was sham-operated (*10*). On the seventh postoperative day, the T/S ratios for the thyroid gland and for the tumor were significantly lower in the hypophysectomized mice than in the controls (table 4).

3) *Effect of feeding propylthiouracil in the diet.*—Mice bearing tumors of independent line 3C were fed the stock diet for 111 days. They were then divided into two groups, one fed the low-iodine diet, the other fed the low-iodine diet supplemented with propylthiouracil to 0.03 percent.

TABLE 4.—*Effect of various factors on the ratio of tissue radioiodide concentration to serum radioiodide concentration**

Purpose	Tumor line	Group	Diet	Days fed low-iodine diet	Tissue radioiodide concentration / Serum radioiodide concentration	
					Thyroid gland	Tumor
Effect of tumor on iodide concentrating by thyroid gland.	4H	No tumor	Thiouracil	0	187 ± 17† (6)‡	None
		Tumor		0	205 ± 3 (6)	105 ± 16 (6)
Effect of days fed stock diet after chronic thiouracil feeding.	4J		Thiouracil	7	256 ± 16 (6)	61 ± 6 (6)
				21	165 ± 12 (6)	58 ± 5 (6)
Effect of hypophysectomy	4H	Sham-operated	Thiouracil	7 + 7§	162 ± 36 (6)	48 ± 6 (6)
		Hypophysectomized		7 + 7§	60 ± 4 (6)	30 ± 1 (5)
Effect of feeding propylthiouracil.	3C	No propylthiouracil	Stock	14	120 ± 19 (7)	0.42 ± .02 (7)
		0.03% propylthiouracil in diet 14 days.	Stock and PTU.	14	283 ± 57 (5)	0.42 ± .01 (5)

*Organic binding blocked by a single injection of 2.5 mg. of propylthiouracil.
†Standard error of the mean.
‡Number of determinations.
§Fed low-iodine diet for 14 days preceding experiment, operation on eighth day.

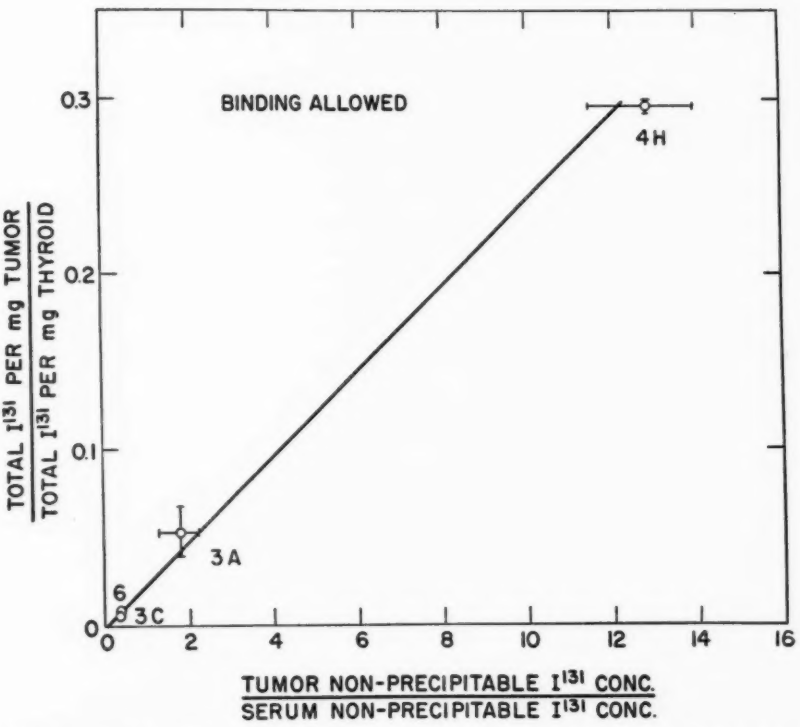
The propylthiouracil feeding had no effect on the T/S ratio for the tumors, although it more than doubled the T/S ratio for the thyroids (table 4).

Discussion

When organic binding of iodide was blocked, the ability of tumors to concentrate radioiodide was less than that of the thyroid gland. The concentration of radioiodide in tumor tissue was much higher than that in serum only in dependent tumors. There was a suggestion that the loss of iodide-concentrating ability occurred during the transition from dependent to independent tumors, since some tumors of line 3A had no ability to concentrate iodide in the generation in which independent tumors first appeared.

When binding was permitted, the T/S ratio was almost as high for tumors of dependent line 4H as for thyroid gland, but the T/S ratio was significantly less for tumors of all other lines than for thyroid glands. In tumors of independent lines 3C and 6, the ability to maintain a concentration of iodide elevated above that of serum was lacking. The T/S ratio in these two lines was essentially what might be expected for several nonthyroid tissues (*8*). Since the T/S ratio was approximately 0.4, the iodide in these tumors would appear to be in large part extracellular.

Although tumors of all lines reported in this paper incorporated iodide into organic binding (table 2), the amount of I^{131} bound per mg. of tissue was always less than that in the thyroid gland (table 3). The concentration of radioiodide in tumors of dependent line 4H when binding was permitted was approximately equal to that in the thyroid gland, but organic bound I^{131} per mg. of tissue was less in the tumor than in the thyroid gland. This suggests that the decreased accumulation of I^{131} in tumors of this line was due to an impairment in the binding mechanism. On the other hand, in the other lines the radioiodide concentration in the tumor was always much less than that in the thyroid gland of the host. In this case the low concentration of radioiodide in the tumor may be responsible for at least part of the impairment in iodide binding. To test this a comparison was made of $\frac{\text{uptake per mg. of tumor}}{\text{uptake per mg. of thyroid}}$ with the T/S ratio for the tumor when binding was allowed for four tumor lines (text-fig. 1). The two quantities were roughly linearly related. If it be assumed that organic binding of tissue radioiodide is a first-order reaction (with the rate of binding given by $K_b I_T$ where K_b is the rate constant for binding and I_T is the radioiodide concentration in the tissue), the relation in text-figure 1 suggests that the difference in tumor clearance or I^{131} binding rate may be due to differences in the radioiodide concentrations in the tumors of the different lines. It also suggests that the rate constant for binding may be the same in these tumor lines. Further studies will be necessary to establish this hypothesis. A detailed discussion of a way in which the tissue iodide concentration might control the rate of organic binding of I^{131} is given elsewhere (*9*).



TEXT-FIGURE 1.—The relation between I^{131}-accumulating ability and radioiodide-concentrating ability of thyroid tumors when organic binding of iodine is allowed. The lines through the points represent standard errors of the mean.

In the preceding paragraph it was indicated that the very low radioiodide-concentrating ability of independent tumors might be an important factor limiting their I^{131} uptake. It is therefore important to study the factors that affect this process, and especially to try to increase the radioiodide-concentrating ability in tumors in which the process limits I^{131} uptake. In the tumor-free mouse, the T/S ratio is reduced but generally does not fall below 50 following hypophysectomy (*4*, *10*). In tumors of the dependent line 4H, the radioiodide-concentrating ability was also decreased following hypophysectomy, but the magnitude of the response was less than that of the thyroid gland (table 4). On the other hand, feeding propylthiouracil did not induce radioiodide-concentrating ability in tumors of independent line 3C (which had lost the ability to concentrate iodide), although it increased that of the thyroid gland in the same host.

When thyroid tumor tissue becomes independent it appears to lose its ability to perform biochemical functions characteristic of thyroid tissue. With respect to the two functional characteristics studied so far, three different types of thyroid tumor tissue have been found: *a*) tumors that

can concentrate iodide and incorporate it into organic binding (lines 4HJK, 3A, and 4); *b*) tumors that cannot concentrate iodide but can bind it (independent tumor lines 3C and 6); and *c*) tumors that can neither concentrate nor bind iodide [independent line 4F (*3*)]. A fourth type of thyroid tissue, one that appears to differ from the above three, is the thyroid gland of the 7-day chick embryo. As shown by Wollman and Zwilling (*11*) 7-day embryonic thyroids had a very well developed ability to concentrate iodide, but no organic binding of iodide was detected. These observations suggest that concentrating and organic binding of iodide are independent processes [6] and that independent thyroid tumors differ from embryonic thyroid gland with respect to their ability to perform these two functions of thyroid tissue.

Administration of propylthiouracil prevented practically all binding of radioiodide in tumors of the thyroid gland. Almost all the I^{131} found in the precipitate after deproteinization could be accounted for on the basis of the volume of the precipitate. It has been reported that thiouracil will allow binding of some I^{131} by tumors of the thyroid gland in man (*12*, *13*) and in the rat (*14*) under conditions where the thiouracil prevents binding of I^{131} by normal thyroid tissue. The present results give no information about whether the concentration of propylthiouracil required for a complete blocking of binding is different for tumors and thyroid gland in mice.

Summary

The ratio of the tissue radioiodide concentration to serum radioiodide concentration (T/S ratio) was measured in several lines of transplantable tumors of the thyroid gland. The T/S ratio was determined both when organic binding of radioiodide was permitted and when it was blocked by propylthiouracil. The T/S ratio was much higher in dependent tumors (the growth of which required the host to be fed thiouracil) than in independent tumors, but it was never as high in dependent tumors as in thyroid gland. In independent tumors the T/S ratio was rarely above two.

Propylthiouracil feeding increased the T/S ratio of the host's thyroid gland but had no effect on the T/S ratio of tumors of an independent line which had lost all iodide-concentrating ability. Hypophysectomy resulted in a reduction of the T/S ratio in tumors of a dependent line.

Some tumors that have lost the ability to maintain a concentration of radioiodide elevated above that of serum have retained ability to incorporate radioiodide into organic binding.

References

(*1*) Morris, H. P., and Green, C. D.: The role of thiouracil in the induction, growth and transplantability of mouse thyroid tumors. Science 114: 44–46, 1951.

[6] Although these processes may occur independently, in the kinetics of I^{131} accumulation they are consecutive, and the rate of binding depends on the concentration of thyroid iodide maintained by the concentrating process as described earlier.

(2) Morris, H. P., Dalton, A. J., and Green, C. D.: Malignant thyroid tumors occurring in the mouse after prolonged hormonal imbalance during the ingestion of thiouracil. J. Clin. Endocrinol. 11: 1281–1295, 1951.

(3) Wollman, S. H., Scow, R. O., Wagner, B., and Morris, H. P.: Radioiodine uptake by transplantable tumors of the thyroid gland in C3H mice. I. Experimental results. J. Nat. Cancer Inst. 13: 785–805, 1953.

(4) Wollman, S. H., and Scow, R. O.: The effect of propylthiouracil on radioiodide concentrating by the thyroid gland in normal and hypophysectomized mice. Endocrinology 53: 332–341, 1953.

(5) Taurog, A., Tong, W., and Chaikoff, I. L.: Non-thyroglobulin iodine of the thyroid gland. II. Inorganic iodide. J. Biol. Chem. 191: 677–682, 1951.

(6) Wollman, S. H., Morris, H. P., and Green, C. D.: Function of transplantable tumors of the thyroid gland in C3H mice. J. Nat. Cancer Inst. 12: 27–35, 1951.

(7) Wollman, S. H.: Radioiodine uptake by transplantable tumors of the thyroid gland in C3H mice. II. Theory. J. Nat. Cancer Inst. 13: 807–814, 1953.

(8) Stevens, C. D., Meinken, M. A., Quinlin, P. M., and Stewart, P. H.: The distribution of radioactive iodine in mice with and without tumor 15091a after injection of radioactive sodium iodide. Cancer Research 10: 155–161, 1950.

(9) Wollman, S. H.: A thyroid model describing the kinetics of exchange, concentrating and organic binding of iodide by thyroid tissue. Endocrinology: In press.

(10) Wollman, S. H., and Scow, R. O.: Effect of hypophysectomy on I^{131} metabolism in normal and thiouracil treated C3H mice. Endocrinology 52: 338–345, 1953.

(11) Wollman, S. H., and Zwilling, E.: Radioiodine metabolism in the chick embryo. Endocrinology 52: 526–535, 1953.

(12) Rawson, R. W.: Radioactive iodine: Its use in studying certain functions of thyroid tumors. Brookhaven Conference Report (Symposium on Radioiodine, pp. 4–12, 1948).

(13) Dobyns, B. M., and Maloof, F.: The study and treatment of 119 cases of carcinoma of the thyroid with radioactive iodine. J. Clin. Endocrinology 11: 1323–1360, 1951.

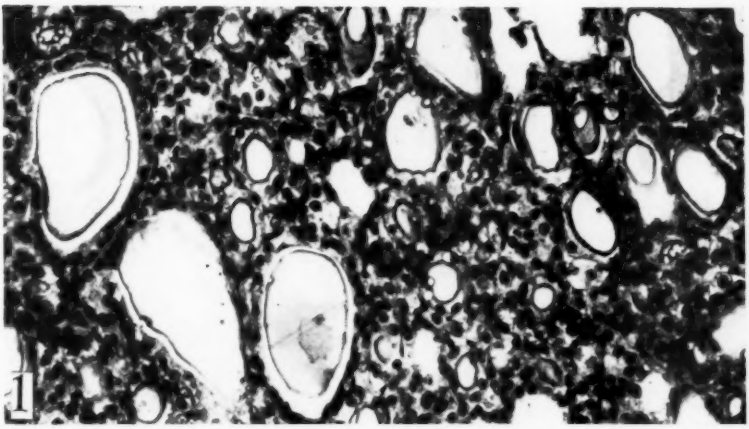
(14) Money, W. L., Fitzgerald, P. J., Godwin, J. T., and Rawson, R. W.: The effect of thiouracil on the collection of radioactive iodine in experimentally induced thyroid tumors. Cancer 6: 111–120, 1953.

PLATE 47

FIGURE 1.—Tumor line 3A. The section was obtained from one of the smaller tumors which resembles those of line 4HJK. Hematoxylin and eosin. × 230

FIGURE 2.—Tumor line 3A. The section was obtained from one of the larger tumors which resembles those of line 3C. Hematoxylin and eosin. × 230

FIGURE 3.—Tumor line 4. Hematoxylin and eosin. × 260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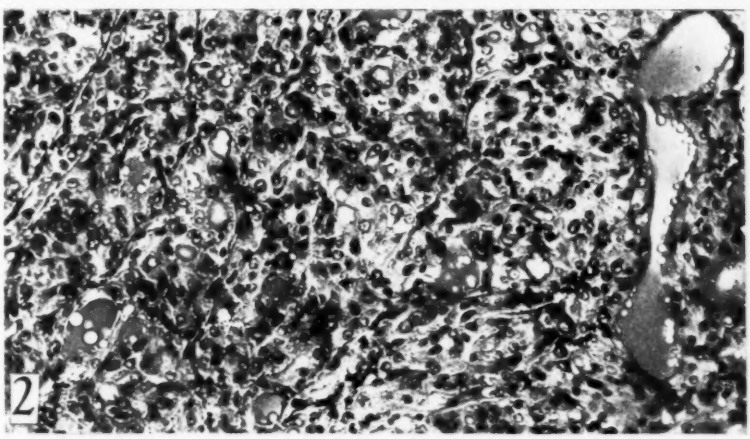
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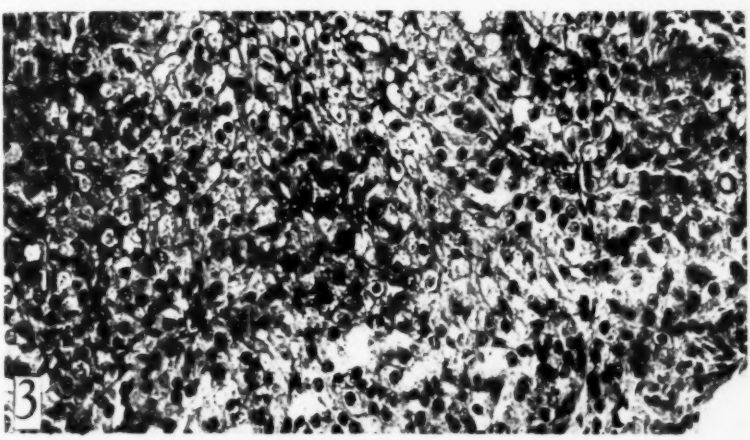
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Tumor-Damaging Capacity of Plant Materials. V. Miscellaneous Plants [1]

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Previous communications (*1–4*) have presented results of a plant survey to determine whether new natural products could be found that would have tumor-necrotizing properties. These plants were usually grouped according to a stated pharmacologic or medicinal action. The alleged uses of many of the plants reported in this paper, however, are so diverse that they have been grouped according to botanical classification. The effects of approximately 150 plant materials upon Sarcoma 37 in mice are given here. Fifty-five botanical families are represented.

Materials and Methods

Materials and methods of testing have been described in detail in the first paper of this series (*1*). As before, the tumor used was Sarcoma 37, implanted in the thigh muscle of CAF_1 mice. A single subcutaneous injection of the plant material in mice bearing six-day-old tumors was given at a site contralateral to the tumor. In the initial experiments, 15 mice were used for each test, 5 animals being autopsied at each of three intervals (7, 24, and 48 hours respectively) after the injection. In later experiments the 7-hour autopsy period was omitted, inasmuch as the 24- and 48-hour autopsy periods were sufficient to give the information desired. Equal numbers of tumor-bearing mice were used for controls. The tumors were observed grossly and histologically for evidence of induced damage; the histologic specimens were routinely fixed in Zenker's formol and stained with hematoxylin and eosin.

Most of these plants were obtained from commercial sources as dry, powdered materials. Aqueous and olive-oil suspensions were tested of all powdered samples and in many cases, where sufficient material was available, alcohol and acid extracts were prepared as previously described (*1*).

Since the cucurbits, bryonia and colocynth, had previously been found to possess tumor-necrotizing activity (*1*), other genera of this family commonly used as foods have now been tested including pumpkin, cucumber, and a number of squashes and melons. These cucurbits were

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obtained as fresh ripe fruits, and hence required somewhat different treatment than the powdered plants. Fruits containing abundant water, *e.g.* melons, were pressed to express the juice; the seeds were discarded. When possible, the juice was taken to dryness by lyophilization; in other cases it was concentrated by the same process to a thick syrup. The pulp was dried at room temperature, ground to a powder, and tested in the same manner as other powdered products.

Preliminary toxicity determinations in normal mice served as a guide for selection of the dose to be used for testing in mice implanted with tumor. A dose sublethal for normal mice occasionally caused 10 to 20 percent deaths in tumor-bearing mice. One mg. per gm. was chosen as the highest dose of powdered material when toxicity was not a factor; 0.04 cc. per gm. was the highest dose of fluid preparations employed.

Results

Results obtained with 150 plant materials are given in table 1. Active materials were retested; new extracts were made for the second test as it could not safely be assumed that the extracts would remain stable.

Of the 16 members of the family *Cucurbitaceae* obtained in fresh form, only a few showed any evidence of tumor-necrotizing capacity. These were in most cases negative when the dried materials were retested one year later (table 1).

Of the other plants tested, only 5 showed any activity in aqueous or olive-oil suspension. These were:

Symplocarpus foetidus
Polygala senega root
Aconitum napellus root
Gillenia stipulata root (one sample only)
Daphne mezereum bark

In all cases but one (aconite root), activity was also demonstrated by either, or both, the acid and alcohol extractions.

Other plants showing some activity in extracts only were:

Marsdenia condurango bark (acid extract only)
Arnica montana flowers (acid extract only)
Brauneria pallida root (alcohol and acid extract)
Gaultheria procumbens herb (alcohol extract only)
Marrubium vulgare herb (alcohol extract only)
Thymus serpyllum (alcohol and acid extract)
Trifolium pratense tops (alcohol extract only)
Liriodendron tulipifera bark (acid extract only)
Prunus americana bark (alcohol and acid extracts)
Cephaelis acuminata root (acid extract only)
Pimpinella saxifraga root (alcohol extract mainly)

Inconsistent results were obtained with skunk cabbage (*Symplocarpus foetidus*) when given as an aqueous suspension, but the alcohol extract was consistently active at doses of 0.005 cc. to 0.01 cc. per gm. A sample of skunk cabbage root had no activity.

Of three samples of *Gillenia stipulata* obtained from the same commercial source, only one showed any activity against Sarcoma 37 in aqueous and olive-oil suspension (at 0.5 mg./gm.). No extracts were made of the samples found inactive in aqueous suspension. The active sample was effective in alcohol extract at 0.0002 cc. per gm.; this, however, was a near-lethal dose.

Discussion

Reports on a number of the plants tested by us have appeared in the literature [4] as to their use in treating tumors, warts, etc., either by local application or parenteral administration. No correlation was found between such reports and the effect of these plants on Sarcoma 37 in mice under the conditions of these experiments.

In some cases activity was confined to plants in only one genus, as in the family *Pinaceae* (*4*) where of 15 genera tested only certain members of the genus *Juniperus* showed activity against Sarcoma 37. As discussed previously, activity sometimes depends on the age of the plant, the time of year collected, or the geographical location in which it is grown (*4*). Possibly this accounts for the discrepancy in results of different samples of the same plant material, *e.g.*, *Gillenia stipulata*.

Since these plants were commercially obtained as powdered materials, no check could be made as to the accuracy of their species designation, time of year collected, or area where they were grown.

The literature offers certain information with regard to the possible carcinolytic constituents of several of the plants showing activity, as for example *Trifolium pratense* (active in alcohol extract only). Nakaoki (*8*) found that a number of phenolic substances isolated from this plant, including pratol and pratensol, and the glycosides trifoliin and isotrifoliin yield quercetin on hydrolysis. Quercetin has been found by Leiter *et al.* (*9*) to cause hemorrhage and necrosis in Sarcoma 37 at high doses (MED 0.2 mg./gm.; MTD 0.6 mg./gm.). None of the four parent substances has been tested as far as we know. Ice and Wender (*10*) found quercetin in the leaves of *Vaccinium myrtillus*. Our test on Sarcoma 37 showed only a questionable effect in one of three samples of this plant.

Ipecac root, cartagena (*Cephaelis acuminata*), which in acid extract produced hemorrhage and necrosis in more than 50 percent of the tumors, is a source of emetine, also found active against Sarcoma 37 by Hartwell and co-workers (*11*). However, another species, Rio ipecac root (*Cephaelis ipecacuanha*), showed no activity in our tests. Indian physic root, *Gillenia stipulata*, is commonly known as American ipecac and is used as a substitute for ipecac (*5*) but is not reported to contain emetine. Related compounds might however be present, accounting for the slight activity found in one of the three samples.

A number of alkaloids derived from plants reported in this series have been tested by Leiter *et al.* in this Laboratory (personal communication).

[4] In the United States Dispensatory (*5*) the following plants are mentioned as having been used against tumors: Condurango bark [Fam. *Asclepiadaceae*, also see (*6*) and (*7*)]; Echinacea root (Fam. *Compositae*); Poke root (Fam. *Phytolaccaceae*); Solomon's seal (Fam. *Liliaceae*).

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials*

Botanical name	Common name and material used	Preparation*	Dose per gram	Effect†
AQUIFOLIACEAE				
Ilex paraguariensis A. St. Hilaire	Paraguay tea, leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
ARACEAE				
Acorus calamus L.	Calamus root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Arisaema triphyllum (L.) Schott	Wild turnip root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Symplocarpus foetidus (L.) Nutt	Skunk cabbage	aq.	1.0 mg.	10/15; 3/20; neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	5/15
			0.01 cc.‡	10/10
		HCl	0.02 cc.	7/15
			0.03 cc.‡	neg.
"	Skunk cabbage root	aq.	0.5 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.01 cc.	neg.
ARALIACEAE				
Aralia racemosa L.	Spikenard root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
ASCLEPIADACEAE				
Asclepias galioides H. B. K.	Milkweed latex	dialysate	0.04 cc.	neg.
		non-dial.	0.005 cc.	neg.
		o. o.	0.02 cc.	neg.
Asclepias sp.	Milkweed root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Asclepias tuberosa L.	Pleurisy root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Marsdenia condurango Reichenbach	Condurango bark	aq.	0.5 mg.	neg.
		o. o.	0.75 mg.	neg.
		EtOH	0.5 mg.	neg.
		HCl	0.04 cc.	2/15; 8/15
"	Condurango bark solid ext.	aq.	1.0 mg.	neg.

BERBERIDACEAE				
Berberis, section *Mahonia* sp.	Berberis root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Caulophyllum thalictroides (L.) Michaux	Blue Cohosh root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	1.0 mg.	neg.
		HCl	0.01 cc.	neg.
BETULACEAE				
Alnus serrulata Willd	Tag alder bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
BURSERACEAE				
Commiphora molmol Engler & other sp.	Myrrh gum	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
"Resina elemi"	Elemi gum	o. o.	1.0 mg.	neg.
CARYOPHYLLACEAE				
Saponaria officinalis L.	European soap root	aq.	0.01 mg.	neg.
		o. o.	0.01 mg.	neg.
COMPOSITAE				
Anacyclus pyrethrum DC.	Pellitory root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.01 cc.	neg.
Arnica montana L. & other sp.	Arnica flowers	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.02 cc.	6/15
			0.03 cc.‡	3/10
Arnica sp.	Arnica root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Brauneria pallida (Nutt.) Britton or *B. angustifolia* (DC) Heller	Echinacea root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	3/9; 4/10
		HCl	0.02 cc.	6/9
			0.01 cc.‡	2/9

*aq. = aqueous suspension; o. o. = olive-oil suspension; EtOH = ethyl alcohol extract; HCl = extraction of residue of alcohol with HCl.

†Expressed as: $\frac{\text{No. mice with induced tumor damage}}{\text{No. mice sacrificed}}$. Untreated control tumors showed no induced damage.

‡New extract.

§According to: Ullman, S. B., Halberstaedter, L., and Leibowitz, J.: Exper. Med. *E* Surg. 3: 11–23, 1945.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials*—Continued

Botanical name	Common name and material used	Preparation*	Dose per gram	Effect†
COMPOSITAE—Continued				
Senecio aureus L.	Life root plant	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Tanacetum vulgare L.	Tansy herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
CUCURBITACEAE				
Citrullis vulgaris Schrad.	Watermelon (fresh) rind	aq.	1.0 mg.	2/15; neg. (1 yr. later)
	red pulp	aq.	1.0 mg.	neg.
	conc. juice of rind	syrup	0.02 cc.	neg.
	" " of red pulp	syrup	0.02 cc.	neg.
Cucumis melo var. *cantalupensis*	Cantaloupe, fresh pulp	aq.	1.0 mg.	neg.
	lyo. juice	aq.	1.0 mg.	neg.
	conc. juice	syrup	0.04 cc.	11/15
			0.04 cc.	5/15 (same prep. 1 week later)
	Frozen cantaloupe	syrup	0.01 cc.	neg.
Cucumis melo var.	Honeydew melon, pulp	aq.	1.0 mg.	neg.
	lyo. juice	aq.	1.0 mg.	neg.
Cucumis sativa L.	Cucumber, pulp	aq.	1.0 mg.	neg.
	lyo. juice	aq.	1.0 mg.	neg.
Cucumis myriocarpus Naud.	Paddy melon, fruit, dried	aq.	0.4 mg.	8/15; 6/7
	root	aq.	0.5 mg.	neg.
	stems & leaves	aq.	1.0 mg.	3/15; 4/8
Cucurbita lagenaria L.	Bottle gourd	aq.	1.0 mg.	neg.
Cucurbita moshata var.	Cushaw squash	aq.	1.0 mg.	neg.
Cucurbita pepo var.	Acorn squash	aq.	1.0 mg.	neg.
" " "	Cymling squash pulp	aq.	1.0 mg.	neg.
" " "	Summer squash, lyo. juice	aq.	1.0 mg.	neg.
" " "	" " " pulp	aq.	1.0 mg.	neg.
" " "	Pumpkin	aq.	1.0 mg.	neg.
" " var. ovifera Bailey	Gourd No. 2	aq.	1.0 mg.	5/14
		aq.	0.5 mg.	neg. (1 yr. later)
" " var.	Gourd No. 1	aq.	1.0 mg.	neg.
" " "	Gourd No. 3	aq.	1.0 mg.	3/13
		aq.	0.5 mg.	neg. (1 yr. later)

" " "	Gourd No. 5	aq.	1.0 mg.	neg.
Momordica charantia L.	Balsam apple, fruit	aq.	1.0 mg.	3/10
		aq.	0.5 mg.	3/10 (1 yr. later)
	leaves & vine	aq.	1.0 mg.	2/15; 2/10 (1 yr. later)
CYCADACEAE				
Cycas revoluta Thunb	Sago palm leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
ERICACEAE				
Gaultheria procumbens L.	Wintergreen herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	4/13; 5/10
		HCl	0.02 cc.	neg.
Vaccinium species	Myrtle herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	2/11
			0.005 cc.‡	neg.
		HCl	0.02 cc.	2/15
			0.01 cc.‡	neg.
Vaccinium myrtillus L. & other sp	Huckleberry leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.02 cc.	neg.
Vaccinium sp	Imported myrtle leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.02 cc.	neg.
Vaccinium sp	Myrtillin concentration	aq.	1.0 mg.	neg.
EUPHORBIACEAE				
Stillingia sylvatica L.	Stillingia root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.01 cc.	neg.
FUCACEAE				
Fucus vesiculosis L.	Bladder wrack	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.

*aq.=aqueous suspension; o. o.=olive-oil suspension; EtOH=ethyl alcohol extract; HCl=extraction of residue of alcohol with HCl.

†Expressed as: $\frac{\text{No. mice with induced tumor damage}}{\text{No. mice sacrificed}}$. Untreated control tumors showed no induced damage.

‡New extract.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials*—Continued

Botanical name	Common name and material used	Preparation*	Dose per gram	Effect†
FUMARIACEAE				
Dicentra canadensis (Goldie) Walpers	Turkey corn	aq.	1.0 mg.	neg.
& *D. cucullaria* (L.) Bernhardi		o. o.	1.0 mg.	neg.
GOODENIACEAE				
Scaevola frutescens (Mill.) Krause	Leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg	neg.
HIPPOCASTANACEAE				
Aesculus hippocastanum L.	Horse chestnut bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.01 cc.	neg.
HYDROPHYLLACEAE				
Eriodictyon californicum (Hooker & Arnott) Torrey	Yerba santa	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
HYPERICACEAE				
Hypericum perforatum L.	Saint John's wort herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	3/14
		HCl	0.02 cc.	neg.
IRIDACEAE				
Iris florentina L.	Florentine orris root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Iris germanica L. & *I. pallida* Lamarck	Verona orris root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Iris sp.	Pinhead orris root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
LABIATAE				
Marrubium vulgare L.	Horehound herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
			0.01 cc.	4/10

		HCl	0.02 cc.	3/8
			0.03 cc.‡	4/9
Salvia officinalis L.	Dalmatian sage	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.02 cc.	neg.
Sideritis sp.	Sideritis herb	aq.	0.5 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.02 cc.	neg.
Thymus serpyllum L.	Wild thyme	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	6/8
			0.01 cc.‡	4/10
		HCl	0.02 cc.	4/10; neg.
LECYTHIDACEAE				
Careya australis F. Muell.	Cocky apple, stems	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
"	Cocky apple, leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
LEGUMINOSAE				
Baptisia tinctoria (L.) R. Brown	Wild indigo root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.005 cc.	neg.
Ichyomethia piscipula Hitchc.	Jamaica dogwood bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.02 cc.	2/15
Medicago sativa L.	Alfalfa herb	aq.	0.5 mg.	neg.
		o. o.	0.5 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.0025 cc.	neg.
Myroxylon pereirae Royle	Peru balsam		0.03 cc.	neg.

*aq.=aqueous suspension; 0.0.=olive-oil suspension; EtOH=ethyl alcohol extract; HCl=extraction of residue of alcohol with HCl.

†Expressed as: $\frac{\text{No. mice with induced tumor damage}}{\text{No. mice sacrificed}}$. Untreated control tumors showed no induced damage.

‡New extract.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials*—Continued

Botanical name	Common name and material used	Preparation*	Dose per gram	Effect†
LEGUMINOSAE—Continued				
Trifolium pratense L.	Red clover tops	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	3/9; 3/9
		HCl	0.02 cc.	neg.
LILIACEAE				
Aletris farinosa L.	Aletris root	aq.	0.25 mg.	neg.
		o. o.	1.0 mg.	neg.
Chamaelirium luteum (L.) A. Gray	Helonius root	aq.	0.5 mg.	neg.
		o. o.	0.5 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.02 cc.	neg.
Convallaria majalis L.	Lily of the Valley leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Polygonatum officinale (L.) All.	American Solomon Seal root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Smilax aristolochiaefolia Miller or *S. ornata* Hook	Sarsaparilla root, Central America	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Smilax rugelli Killip & Morton	Sarsaparilla root, Honduras	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Urginea indica Kunth	White Indian squill	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.0013 cc.	neg.
		HCl	0.02 cc.	neg.
Urginea maritima (L.) Baker	White Mediterranean squill	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.0025 cc.	neg.
		HCl	0.03 cc.	neg.
LOBELIACEAE				
Lobelia inflata L.	Lobelia leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.

LORANTHACEAE				
Viscum album L.	Mistletoe herb, foreign	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.01 cc.	neg.
Viscum flavescens Pursh	Mistletoe herb, American	aq.	0.75 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.01 cc.	neg.
MAGNOLIACEAE				
Liriodendron tulipifera L.	Poplar bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.02 cc.	9/14; 1/10
Magnolia virginiana L.	Magnolia bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.02 cc.	neg.
MALVACEAE				
Gossypium herbaceum L. & other sp.	Cotton root bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Thespesia populnea Corr.	Bark	aq.	1.0 mg.	neg.
MELIACEAE				
Guarea rusbyi (Britton) Rusby	Cocillana bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
MENISPERMACEAE				
Anamirta cocculus (L.)	Cocculus indicus	aq.	0.1 mg.	neg.
		o. o.	0.2 mg.	neg.
		EtOH	0.2 mg.	neg.
		HCl	0.03 cc.	neg.
Menispermum canadense L.	Yellow parilla root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
MONIMACEAE				
Boldu boldus (Molina) Lyons	Boldo leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.

*aq.=aqueous suspension; o.o.=olive-oil suspension; EtOH=ethyl alcohol extract; HCl=extraction of residue of alcohol with HCl.

†Expressed as: $\frac{\text{No. mice with induced tumor damage}}{\text{No. mice sacrificed}}$. Untreated control tumors showed no induced damage.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials*—Continued

Botanical name	Common name and material used	Preparation*	Dose per gram	Effect†
MORACEAE				
Humulus lupulis L.	Lupulin	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
"	Humulon crystals	aq.	0.3 mg.	neg.
MYRTACEAE				
Eucalyptus globulus Labillardiere	Eucalyptus leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
OLEACEAE				
Fraxinus americana L.	White ash bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Fraxinus nigra Marsh	Black ash bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Jasminum simplicifolium Forst	Stems and leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
PASSIFLORACEAE				
Passiflora incarnata L.	Passion flower herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
PHYTOLACCACEAE				
Phytolacca americana L.	Poke root	aq.	0.75 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.5 mg.	neg.
		HCl	0.005 cc.	neg.
"	Phytolaccin concentrate	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
"	Poke berry, fresh green	aq.	0.0013 cc.	neg.
		EtOH	0.0006 cc.	2/15
		HCl	0.01 cc.	neg.
PIPERACEAE				
Piper banksii	Stems and leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.

PLUMBAGINACEAE				
Plumbago zeylanica L.	Stems and leaves	aq.	1.0 mg.	neg.
"	Root	aq.	1.0 mg.	neg.
POLYGALACEAE				
Polygala senega L.	Senega root	aq.	0.05 mg.	3/15; 4/9
		o. o.	0.05 mg.	neg.
		EtOH	0.0025 cc.	8/15
			0.0001 cc.‡	4/8
		EtOH ppt.	1.0 mg.	4/4
			0.5 mg.	4/4
		HCl	0.0006 cc.	3/15
			0.0025 cc.‡	2/10
POLYPODIACEAE				
Cheilanthes sieberi	Stems and leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
PRIMULACEAE				
Anagallis arvensis L.	Scarlet pimpernel plant	aq.	0.125 mg.	neg.
		o. o.	1.0 mg.	neg.
RANUNCULACEAE				
Aconitum napellus L.	Aconite leaves	aq.	0.25 mg.	neg.
		o. o.	0.25 mg.	neg.
		EtOH	0.0004 cc.	neg.
		HCl	0.002 cc.	neg.
"	Aconite root	aq.	0.075 mg.	4/15
		o. o.	0.125 mg.	neg.
		EtOH	0.000033 cc.	neg.
		HCl	0.0006 cc.	neg.
Helleborus niger L.	Black hellebore root	aq.	0.2 mg.	neg.
		o. o.	0.4 mg.	neg.
		EtOH	0.0006 cc.	neg.
		HCl	0.0006 cc.	neg.
Hydrastis canadensis L.	Golden seal herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.

*aq.=aqueous suspension; 0.0.=olive-oil suspension; EtOH=ethyl alcohol extract; HCl=extraction of residue of alcohol with HCl.

†Expressed as: $\frac{\text{No. mice with induced tumor damage}}{\text{No. mice sacrificed}}$. Untreated control tumors showed no induced damage.

‡New extract.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials*—Continued

Botanical name	Common name and material used	Preparation*	Dose per gram	Effect†
RHAMNACEAE				
Ceanothus americanus L.	Jersey tea root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
"	Jersey tea bark of root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
ROSACEAE				
Gillenia stipulata (Muhl.) Trel.	Indian physic root (sample A)	aq.	0.5 mg.	5/15; 10/15
		o. o.	0.5 mg.	2/15
		EtOH	0.0002 cc.	8/14; 3/10
		HCl	0.01 cc.	4/15
			0.02 cc.‡	1/9
	(sample B)	aq.	0.5 mg.	neg.
	(sample C)	aq.	0.5 mg.	neg.
Prunus americana Marsh.	Wild plum bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	4/10; neg.
		HCl	0.02 cc.	4/10
			0.01 cc.‡	2/10
Prunus serotina Ehrhart	Wild cherry bark (sample A)	aq.	1.0 mg.	2/15
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	2/10
		HCl	0.03 cc.	neg.
	(sample B)	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
	(sample C)	aq.	1.0 mg.	neg.
Prunus spinosa L.	Sloe berries	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Quillaja saponaria Molina	Soap bark	aq.	0.1 mg.	neg.
		o. o.	0.1 mg.	neg.
		EtOH	0.0025 cc.	neg.
		HCl	0.0005 cc.	neg.

RUBIACEAE				
Cephaelis acuminata Karsten	Ipecac root, Cartagena	aq.	0.25 mg.	neg.
		o. o.	0.5 mg.	neg.
		EtOH	0.0013 cc.	neg.
		HCl	0.005 cc.	10/15; 5/10
Cephaelis ipecacuanha (Brotero)	Ipecac root, Rio	aq.	0.5 mg.	neg.
		o. o.	0.5 mg.	neg.
		EtOH	0.0025 cc.	neg.
		HCl	0.005 cc.	neg.
RUTACEAE				
Ruta graveolens L	Rue herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Zanthoxylum americanum Miller	Northern prickly ash bark	aq.	0.5 mg.	neg.
		o. o.	1.0 mg.	neg.
" & *Z. clava-herculis* L.	Prickly ash berries	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Zanthoxylum clava-herculis L.	Southern prickly ash bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.01 cc.	neg.
SALICACEAE				
Populus candicans Aiton or *P. tacamahacca* Miller	Balm gilead buds	EtOH	0.02 cc.	neg.
		HCl	0.03 cc.	neg.
Salix sp.	White willow bark	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.02 cc.	neg.
SOLANACEAE				
Brunfelsiz hopeana Benth	Manaca root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.01 cc.	neg.

*aq.=aqueous suspension; o. o.=olive-oil suspension; EtOH=ethyl alcohol extract; HCl=extraction of residue of alcohol with HCl.

†Expressed as: $\frac{\text{No. mice with induced tumor damage}}{\text{No. mice sacrificed}}$. Untreated control tumors showed no induced damage.

‡New extract.

TABLE 1.—*Damage induced in Sarcoma 37 with plant materials*—Continued

Botanical name	Common name and material used	Preparation*	Dose per gram	Effect†
SOLANACEAE—Continued				
Datura stramonium L.	Stramonium leaves	aq.	0.5 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.01 cc.	neg.
Solanum carolinense L.	Horse nettle berries	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Solanum dulcamara L.	Bittersweet twigs	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	neg.
		HCl	0.01 cc.	neg.
STYRACEAE				
Styrax benzoin Dryander	Sumatra benzoin gum	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Styrax benzoides Craib, *S. tonkinensis* and other sp	Siam type benzoin gum	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
THYMELEACEAE				
Daphne mezereum L.	Mezereon bark	aq.	1.0 mg.	3/15; 3/9
		o. o.	1.0 mg.	neg.
		EtOH	0.0025 cc.	4/14; 9/10
			0.005 cc.‡	6/6
		HCl	0.02 cc.	3/14; neg.
ULMACEAE				
Ulmus fulva Michaux	Elm bark slabs	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Ulmus sp	Elm bark cuttings, southern	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.

UMBELLIFERAE				
Angelica atropurpurea	American angelica root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Conium maculatum L.	Conium leaves	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.02 cc.	neg.
"	Conium seed	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Dorema ammoniacum Don	Ammoniac gum	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Ferula assa-foetida L., *F. foetida* (Bunge) Regal and other sp	Asafoetida	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Ferula sumbul (Kaufmann) Hooker	Musk root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.01 cc.	neg.
		HCl	0.02 cc.	neg.
Petroselinum sativum Hoffman	Green apiol		0.02 cc.	neg.
"	Parsley root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
Pimpinella saxifraga L. and *P. magna* L.	Pimpinella root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
		EtOH	0.005 cc.	6/10; 2/10
		HCl	0.02 cc.	2/10
			0.01 cc.‡	neg.
VALERIANACEAE				
Valeriana officinalis L.	Belgian valerian root	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
VERBENACEAE				
Verbena hastata L.	Vervain blue herb	aq.	1.0 mg.	neg.
		o. o.	1.0 mg.	neg.
ZYGOPHYLLACEAE				
Guajacum officinale L. or *G. sanctum* L.	Guaiac gum	aq.	1.0 mg.	neg.

*aq.=aqueous suspension; o. o.=olive-oil suspension; EtOH=ethyl alcohol extract; HCl=extraction of residue of alcohol with HCl.

†Expressed as: $\frac{\text{No. mice with induced tumor damage.}}{\text{No. mice sacrificed}}$ Untreated control tumors showed no induced damage.

‡New extract.

These include: Berberine, from *Mahonia* sp., and *n*-methylcytisine from *Caulophyllum thalictroides* (Fam. *Berberidaceae*); bulbocapnine, corydine, corlumine, cryptopine, α-allocryptopine and protopine from *Dicentra canadensis* and *D. Cucullaria* (Fam. *Fumariaceae*); lobeline from *Lobelia inflata* (Fam. *Lobeliaceae*); dauricine from *Menispermum canadense* (*Fam. Menispermaceae*); sparteine and 1-ephedrine from *Aconitum napellus*, and hydrastine, berberine and tetrahydroberberine (canadine) from *Hydrastis canadensis* (Fam. *Ranunculaceae*); solanine, from *Solanum dulcamara* (Fam. *Solanaceae*) and coniine from *Conium maculatum* (Fam. *Umbelliferae*). Of these, cryptopine, dauricine, and protopine produced damage in Sarcoma 37; the others were without effect.

Dyer (*7*) in her *Index of Tumor Chemotherapy* lists preparations of aconite, hemlock and ipecac, and also a number of alkaloids derived from plants tested in this series as having been used against various tumors, *viz.*: atropine, emetine, ephedrine, sparteine, cryptopine, and protopine.

Many of the plant preparations stated by Patti (*12*) as ineffective in producing growth inhibition in mouse sarcoma 180, have been used by us [(*1–3*) and table 1, this paper]. The preparations reported by Patti represented aqueous extracts of residues after complete removal of alkaloid fractions.

Various genera of the family *Umbelliferae* were tested because of reports in the literature of two members of this family that contain substances possibly chemically related to silicicolin, a tumor-necrotizing compound isolated in this Laboratory by Hartwell *et al.* (*13*, *14*) from *Juniperus silicicola*. These compounds, anthricin and cicutin, which have the same empirical formula as silicicolin, were isolated by Noguchi and Kawanami (*15*) from *Anthriscus sylvestris* Hoffm. (wild chervil) and by Marion (*16*) from *Cicuta maculata* L. (water hemlock), respectively. Of six additional genera tested, only *Pimpinella saxifraga* showed any tumor-necrotizing activity.

Summary

About 150 plant materials, representing 55 botanical families, were examined for tumor-necrotizing effect, following a single injection, on Sarcoma 37 in CAF_1 mice. Twenty-one plants, or 14 percent of those tested, showed some activity under the conditions employed.

Isolation of the active principles from some of these plants may yield compounds of interest, as was the case with plants examined previously.

Bibliography

(*1*) Belkin, M., and Fitzgerald, D. B.: Tumor-damaging capacity of plant materials. I. Plants used as cathartics. J. Nat. Cancer Inst. 13: 139–155, 1952.

(*2*) Belkin, M., Fitzgerald, D. B., and Felix, M. D.: Tumor-damaging capacity of plant materials. II. Plants used as diuretics. J. Nat. Cancer Inst. 13: 741–744, 1952.

(*3*) Belkin, M., and Fitzgerald, D. B.: Tumor-damaging capacity of plant materials. III. Plants used as pesticides. J. Nat. Cancer Inst. 13: 889–893, 1953.

(*4*) FITZGERALD, D. B., BELKIN, M., FELIX, M. D., and CARROLL, M. K.: Tumor-damaging capacity of plant materials. IV. Conifers. J. Nat. Cancer Inst. 13: 895–903, 1953.

(*5*) United States Dispensatory, 20th ed. Philadelphia, J. B. Lippincott Co., 1918.

(*6*) RUSCHENBERGER, W. S. W.: A report on the origin and therapeutic properties of cundurango. Washington, Government Printing Office, 1873.

(*7*) DYER, H. M.: An Index of Tumor Chemotherapy. Public Health Service, Federal Security Agency. Washington, U. S. Government Printing Office, 1949.

(*8*) NAKAOKI, T.: Glucosides of the flavone series. III. Constituents of *Trifolium repens*. Chem. Abs. 29: 7982, 1935.

(*9*) LEITER, J., DOWNING, V., HARTWELL, J. L., and SHEAR, M. J.: Damage induced in Sarcoma 37 with podophyllin, podophyllotoxin, alpha-peltatin, beta-peltatin and quercetin. J. Nat. Cancer Inst. 10: 1273–1293, 1950.

(*10*) ICE, C. H., and WENDER, S. H.: Quercetin and its glycosides in leaves of *Vaccinium myrtillus*. J. Am. Chem. Soc. 75: 50–52, 1953.

(*11*) HARTWELL, J. L., SHEAR, M. J., JOHNSON, J. M., and KORNBERG, S. R. L. VI. Selection and synthesis of organic compounds. (Abstract.) Cancer Research 6: 489, 1946.

(*12*) PATTI, J.: Mouse Sarcoma 180 inhibition tests. Cancer Research, Suppl. No. 1: 65–67, 1953.

(*13*) HARTWELL, J. L., JOHNSON, J. M., FITZGERALD, D. B., and BELKIN, M.: Silicicolin, a new compound isolated from *Juniperus silicicola*. J. Am. Chem. Soc. 74: 4470, 1952.

(*14*) HARTWELL, J. L., SCHRECKER, A. W., and JOHNSON, J. M.: The structure of silicicolin. J. Am. Chem. Soc. 75: 2138–2140, 1953.

(*15*) NOGUCHI, K., and KAWANAMI, M.: Active components of the *Umbelliferae* X. Components of *Anthriscus sylvestris* Hoffm. Part I. (In Japanese.) J. Pharm. Soc. Japan 60: 629, 1940.

(*16*) MARION, L.: The isolation of cicutin from *Cicuta maculata* L. Canad. J. Research 20B: 157–160, 1942.

Effect of Mengo Encephalitis Virus on the Ehrlich Ascites Tumor of the Mouse [1,2]

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In reports published elsewhere, the tumor-destroying properties of the Bunyamwera virus were described (*1*) and the mechanism of oncolysis was set forth in detail (*2*). In the course of further investigation of the effect of viral infections on the Ehrlich ascites tumor of the mouse, it was discovered that Mengo virus displayed a powerful oncolytic action similar to that of the Bunyamwera virus. The currently described series of experiments was undertaken to confirm the tumor-invading and tumor-destroying properties of the Mengo virus.

Materials and Methods

Virus.—The Mengo encephalitis virus was obtained through the kindness of Dr. K. C. Smithburn of the Laboratories of the Division of Medicine and Public Health of The Rockefeller Foundation, New York. It was maintained in the laboratory through serial passages in brain tissue of Swiss albino mice. The inoculum used in the actual experiments consisted of a 20 percent suspension of infected mouse brain kept frozen at −30° C. Physiologic salt solution was used in making serial dilutions of the viral inoculum.

Tumor.—The Ehrlich ascites tumor was obtained through the kindness of Dr. George Klein of the Karolinska Institute, Stockholm, Sweden. Techniques employed for its maintenance and for the preparation of implants have been described previously (*1*). In the present study, the virus was inoculated intraperitoneally into mice in which the ascites tumor cells had been implanted four days previously.

Mice.—Swiss albino mice, five to eight weeks old, were used throughout the experiments. The virus-inoculated animals were observed for ten days, and the number of sick and dead recorded either once or twice daily.

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Cytologic technique.—The cytologic technique was based on the evaluation of changes in differential counts of cells in smears of ascitic fluid prepared according to Papanicolaou's method. The details of this technique have been described in an earlier communication (*1*).[4]

Experimental Observations

Increased susceptibility of tumor-bearing mice to infection with Mengo virus.—In the preliminary experiments, it was observed that mice bearing ascites tumors became infected and died following inoculation of virus dilutions that had displayed no lethal effect on non-tumor-bearing control mice. This seemingly increased affinity of the virus for tumor cells was further explored in the series of six comparative titration experiments summarized in table 1. In these experiments, mice bearing four-day-old implants of ascites tumors were divided into groups and injected intra-abdominally with aliquots of serial tenfold dilutions of Mengo virus. Simultaneously, the virus was titrated in the normal mice injected by the intra-abdominal or by the intracerebral route. It thus appears from the results of experiments A and B that although the mortality ratios of ascites-tumor-bearing mice may not have been as uniform as those of the controls, the former became infected with virus dilutions that had failed to display a lethal effect upon the control mice.

These differences were particularly striking in the two groups that had been injected parenterally, but even direct intraneural inoculation of the virus in dilutions that were still infectious for ascites-bearing mice failed to elicit a lethal effect. That this effect was not caused solely by the larger volume (0.5 ml.) injected intra-abdominally, as compared with that inoculated intracerebrally (0.03 ml.), was indicated by the results of experiment C. It may be noted that though no titration end points were reached in tumor-bearing mice, 10^{-7} dilution of virus injected in 0.03 ml. volume into ascitic mice caused death of all animals, whereas the same volume of the same dilution of virus failed to cause signs of illness in normal controls injected either intra-abdominally or, which is more significant, intracerebrally. Similar results were obtained in experiment D, and even more conclusively in experiments E and F when titration end points were reached.

Multiplication of the Mengo virus in ascitic fluid.—The results of the preceding experiments indicate that the presence of ascites tumor causes a highly significant increase in the susceptibility of mice to the Mengo virus. It thus becomes evident that one of the components of ascitic fluid made the mice more vulnerable to the lethal action of the virus, and that the neoplastic element seems to be a suitable substrate for multiplication at an increased rate. To obtain additional information, the following experiment was undertaken.

Swiss albino mice were injected intra-abdominally with 12 million cells each of Ehrlich ascites tumor. At the same time another group of mice

[4] Since the inocula prepared from infected ascites tumors invariably caused death of the animals from viral infection, the capacity of the virus-infected neoplastic cells to produce tumors when injected subcutaneously in mice could not be assayed.

TABLE 1.—*Titration of Mengo virus in ascites-tumor-bearing mice and in normal mice*

Experiment	Mice	Inoculation route*	Volume	Mortality ratio following infection with dilutions of Mengo virus								LD_{50} titer of Mengo virus in the ascitic fluid
			(*ml.*)	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	
A	Ascites	i. p.	0.5	.....	5/5	5/5	4/5	2/5	3/5	3/5	0/5	$10^{-9.00}$
	Normal	i. p.	.5	.....	5/5	5/5	2/5	0/5	0/5	0/5	0/5	$10^{-6.85}$
	Normal	i. c.	.03	.....	5/5	5/5	3/5	1/5	0/5	0/5	0/5	$10^{-7.35}$
B	Ascites	i. p.	.5	.....	.....	4/5	3/5	2/5	1/5	.....	.....	$10^{-7.50}$
	Normal	i. p.	.5	.....	5/5	2/5	0/5	0/5	0/5	.....	.....	$10^{-5.85}$
	Normal	i. c.	.03	.....	.....	5/5	3/5	0/5	0/5	.....	.....	$10^{-7.15}$
C	Ascites	i. p.	.03	5/5	5/5	5/5	5/5	.....	.....	.....	.....	$>10^{-7.00}$
	Ascites	i. p.	.5	5/5	5/5	5/5	3/3	.....	.....	.....	.....	$>10^{-7.00}$
	Normal	i. p.	.03	3/3	3/3	3/3	0/3	0/3	0/3	.....	.....	$10^{-6.50}$
	Normal	i. c.	.03	3/3	3/3	3/3	0/3	0/3	0/3	.....	.....	$10^{-6.50}$
D	Ascites	i. p.	.5	5/5	5/5	5/5	5/5	5/5	.....	.....	.....	$>10^{-8.00}$
	Normal	i. p.	.5	5/5	5/5	5/5	1/5	0/5	0/5	.....	.....	$10^{-6.65}$
	Normal	i. c.	.03	5/5	5/5	5/5	1/5	1/5	0/5	.....	.....	$10^{-6.75}$
E	Ascites	i. p.	.5	.....	5/5	4/5	5/5	5/5	1/5	0/5	.....	$10^{-8.50}$
	Normal	i. p.	.5	.....	5/5	5/5	1/5	1/5	0/5	.....	.....	$10^{-6.75}$
	Normal	i. c.	.03	.....	5/5	5/5	4/5	1/5	0/5	.....	.....	$10^{-7.50}$
F	Ascites	i. p.	.5	.....	4/6	5/6	5/6	3/6	1/6	1/6	.....	$10^{-7.65}$
	Normal	i. p.	.5	.....	6/6	0/6	0/6	0/6	0/6	0/6	.....	$10^{-5.50}$
	Normal	i. c.	.03	.....	6/6	6/6	2/6	0/6	0/6	0/6	.....	$10^{-6.65}$

*i. p. = intraperitoneal; i. c. = intracerebral.

was similarly injected with a suspension of Toxoplasma (RH strain) freshly obtained by abdominal puncture of mice infected with this organism. Four days later a few mice in each group were sacrificed and the total quantity of ascitic fluid available from each mouse was aspirated and individually measured; 3.5 ml. of the two types of ascitic fluid were obtained. Smears prepared from the two types of ascites revealed that the non-neoplastic and nonparasitic cellular elements were alike, and consisted mainly of histiocytes, polymorphonuclear leukocytes and lymphocytes. In Toxoplasma ascites, numerous phagocytic cells containing intracellular parasitic organisms were also observed. In the ascites of Ehrlich carcinoma, only rarely did we note histiocytes containing cytoplasmic inclusions of phagocytized material, which consisted of erythrocytes or fragments of broken-down nuclei.

Following this examination, the ascites-bearing mice were divided into several groups, and aliquots of two dilutions of the Mengo virus (see table 2) were injected intra-abdominally. Results of subsequent titration of the virus in normal control mice indicated that the tumor-bearing and the Toxoplasma-ascites-bearing mice received 10 and 100 intraperitoneal LD_{50} doses of virus, respectively.

TABLE 2.—*Comparative rates of multiplication of Mengo and eastern equine viruses in Ehrlich ascites tumor and in Toxoplasma ascites*

Virus inoculum			LD_{50} titer of Mengo virus in the ascitic fluid	
Strain	Dilution of virus suspension	Mouse LD_{50}	Tumor ascites	Toxoplasma ascites
Mengo	10^{-4} 10^{-5}	10 100	$10^{-6.50}$ $10^{-5.50}$	$10^{-2.50}$ $10^{-2.75}$
Eastern	1:5 1:20	....	$10^{-0.75}$ $10^{-0.50}$	$10^{-3.25}$ $10^{-1.60}$

For control purposes, two other groups of ascitic mice were inoculated by the same route with the virus of eastern equine encephalomyelitis. This virus provided an example of an agent which, in marked contrast to Mengo virus, had repeatedly failed to multiply in the Ehrlich ascites tumor (*1*). Again, two dilutions of eastern equine encephalitis virus were used as inoculum (see table 2).

Four days later the majority of mice infected with either of the two viruses sickened, and were accordingly sacrificed. The ascitic fluid was aspirated from each animal and pooled with aliquots of fluid obtained from two other mice in the same experimental group. The eight pools thus secured were centrifuged for 15 minutes at 1,500 r.p.m., and the respective supernatants were alternately frozen and thawed three times. This was done in order to destroy the infectivity of the Toxoplasma, which would otherwise have interfered with the results of subsequent titrations.

Serial tenfold dilutions of the eight pools of ascitic fluids were then made in physiologic salt solution and immediately injected into mice by the intracerebral route. The results of these titrations are summarized in table 2.

An analysis of these results indicates that, although other factors may have influenced the level of multiplication of the Mengo virus in ascitic fluids, the presence of tumor cells is of primary importance. The behavior of the virus in Toxoplasma ascites differs markedly from that observed in tumor ascites. The best example is provided by the titers resulting from inoculation of 100 LD_{50} of the virus: $10^{-5.50}$ in tumor-bearing mice and $10^{-2.75}$ in Toxoplasma-ascites-bearing mice. Similar results were noted in mice receiving 10 LD_{50} of the virus.

In contrast to these results the eastern virus seems to multiply in the Toxoplasma ascites; the neoplastic elements not only fail to support the growth of this virus, but display an apparently inhibitory effect upon the survival of the virus in the peritoneal cavity of the mouse.

Cytologic changes following inoculation of Mengo virus into ascites-tumor-bearing mice.—Preliminary results are summarized in experiments I and II (tables 3 and 4).

In experiment I, serial tenfold dilutions of the virus were inoculated into ascites-bearing mice in 0.5 ml. volumes. The inoculum was simultaneously titrated in normal mice injected intracerebrally (table 3). Three and four days after inoculation the tumor-bearing animals that showed signs of virus infection were tapped and the ascitic fluid aspirated. At the same time ascitic fluid was drawn from noninfected control mice. Fluids from virus-infected mice were obtained in very small quantities and were therefore pooled, two smears from each pool being prepared. The balance of each pool of ascitic fluid was centrifuged for 15 minutes at 1,500 r.p.m., and the supernatant titrated intracerebrally in mice. The resulting LD_{50} titers (last column of table 3) were of high magnitude and indicative of a very high multiplication rate of the virus in tumor tissue.

It may be seen from experiment I, table 4, that the percentage of malignant cells in three control slides was 91, 67, and 76. These cells are shown in figure 1, which illustrates a typical high-power view of a control smear containing numerous discrete malignant cells and only occasional non-neoplastic elements. In mice inoculated with 10^{-5} through 10^{-8} dilutions of the virus, the number of tumor cells varied from 0 to 2 percent, with the exception of one slide in which it rose to 26 percent. In this particular slide, however, tumor cells were not uniformly distributed but were concentrated in a large cluster of 22 cells which focused on a level different from that of the other cells and which most probably represented a "floater," or artifact. Even including this presumably extraneous collection of tumor cells, the resulting count was much below that found in the controls. Mitotic figures were absent from smears of infected mice, and up to 11 percent of the cells showed degenerative changes in contrast to control smears (1 to 3 percent mitoses, 0 percent degenerated cells).

As for non-neoplastic cells, the disappearance of malignant cells resulted

TABLE 3.—*Effect of Mengo virus on ascites tumor in mice (primary data supporting tables 4 and 5)*

Experiment No.	Tumor cells injected (*millions*)	Virus inoculum			Mortality ratio		Mice tapped		LD_{50} titer of Mengo virus in the ascitic fluid
		Dilution of virus suspension	Mouse LD_{50}		Virus	Ascites	Number	Days after virus inoculated	
			intraperitoneal	intracerebral					
I	10	10^{-5}	N. T.*	3, 300	3/3	.....	2	3	$10^{-9.00}$
		10^{-6}	.....	330	3/3	.....	3	3	$10^{-7.80}$
		10^{-7}	.....	33	3/3	.....	3	3	$10^{-9.00}$
		10^{-8}	.....	3	3/3	.....	3	4	$10^{-9.00}$
		Control	.....	.....	.....	4/4	.....	.....	.....
II	12	10^{-4}	450	18, 480	5/5	.....	3	2	$10^{-6.40}$
		10^{-5}	45	1, 848	5/5	.....	2	3	$10^{-6.40}$
		10^{-6}	4. 5	184	5/5	.....	3	3	$10^{-7.85}$
		10^{-7}	≤ 1	18	5/5	.....	4	3	$10^{-7.40}$
		10^{-8}	≤ 1	1	5/5	.....	4	3	$10^{-7.50}$
		Control	.....	.....	.....	6/6	.....	.....	.....
III	12	10^{-2}	56, 230	310, 000	5/5	.....	2	2	$10^{-8.00}$
		10^{-3}	5, 623	31, 000	5/5	.....	2	2	$10^{-7.10}$
		10^{-4}	562	3, 100	5/5	.....	3	2	$10^{-6.60}$
		10^{-5}	56	310	5/5	.....	2	2	$< 10^{-1.00}$
		10^{-6}	5	31	4/5	.....	3	3	$10^{-5.50}$
		10^{-7}	≤ 1	3	5/5	.....	3	3	$10^{-5.50}$
		10^{-8}	≤ 1	≤ 1	5/5	.....	3	3	$10^{-7.50}$
		10^{-9}	≤ 1	≤ 1	1/5	.....	1	4	$< 10^{-1.00}$
		Control	.....	.....	.....	6/6	.....	.....	.....

*N. T.=not titrated.

TABLE 4.—*Cytologic changes in the Ehrlich ascites tumor induced by Mengo virus—experiments I and II (in percentages of differential cell count for each pool of fluid)**

Experiment No.	Virus dilution inoculated	Mice tapped (days after virus inoculated)	Tumor cells			Histiocytes			Polymorphs			Lymphocytes			Mitoses			Degenerated cells			Cells in karyorrhexis			Cells with phagocytic inclusions		
			I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
I	10^{-5}	3	0	1	....	14	1	....	75	84	....	7	13	....	0	0	0	4	1	....	Not counted					
	10^{-6}	3	2	26†	....	2	5	....	67	53	....	18	14	....	0	0	0	11	2	....						
	10^{-7}	3	0	1	....	4	2	....	86	91	....	10	5	....	0	0	0	0	1	....						
	10^{-8}	4	1	0	....	4	2	....	82	76	....	10	13	....	0	0	0	3	9	....						
	Control	3	91	67	76	1	6	5	1	22	14	6	4	2	1	1	3	0	0	0						
II	10^{-4}	1	1	1	1	4	7	4	73	77	32	21	6	63	0	0	0	0	9	0	0	0	+	1	0	0
		2	1	0	X	1	2	....	94	85	....	2	10	....	0	0	....	2	1	....	0	2	....	0	0	....
		3	X	X	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
	10^{-5}	1	48	34	78	14	17	1	19	26	20	13	14	1	0	0	0	4	8	+	2	1	0	0	0	0
		2	0	0	X	43	19	....	47	42	....	10	39	....	0	0	....	0	0	....	0	0	....	0	0	....
		3	X	X	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
	10^{-6}	1	68	32	....	0	6	....	27	40	....	5	9	....	0	0	....	+	12	....	0	1	....	0	0	....
		2	5	1	....	14	1	....	50	40	....	21	57	....	0	0	....	10	1	....	0	0	....	+	0	....
		3	X	X	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
	10^{-7}	1	1	10	90	4	7	6	71	55	2	13	10	1	0	0	1	11	18	0	+	+	0	0	0	0
		2	4	0	4	3	1	7	48	79	44	3	2	15	0	0	0	42	18	30	0	0	0	0	0	0
		3	1	X	X	12‡	....	....	50	....	....	16	....	....	0	....	....	21	....	....	0	....	....	0	....	....
		4	X	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
	10^{-8}	1	87	79	86	6	4	7	6	11	6	1	6	1	0	0	0	0	0	0	0	0	0	0	0	0
		2	79	87	16	3	4	1	7	6	55	3	1	10	2	0	0	5	1	18	1	1	0	0	0	0
		3	1	0	X	7	50	....	79	38	....	7	5	....	0	0	....	6	0	....	0	0	....	0	7	....
		4	X	X	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
	Controls	1	88	76	87	5	14	10	3	7	0	3	2	2	1	1	1	0	0	0	0	0	0	0	0	0
		2	84	69	87	3	14	6	5	15	6	5	1	1	3	1	0	0	0	0	0	0	0	0	0	0
		3	87	65	—	4	25	—	6	9	—	1	0	—	1	0	—	1	0	—	0	0	—	0	1	—

*0=no cells; —=not counted; +=observed in smear but not counted; X=dead; and I, II, III are slide numbers. † Floater. ‡ Epithelioid histiocytes.

in an almost selective response on the part of polymorphonuclear leukocytes, while no marked increase in histiocytes or lymphocytes was observed.

In experiment II, serial tenfold dilutions of Mengo virus were injected into mice bearing ascites tumors and the inoculum was simultaneously titrated by the intraperitoneal and intracerebral routes in normal mice (table 3). Starting from the first day after virus inoculation, tumor-bearing mice, plus a group of noninfected controls, were tapped and sufficient ascitic fluid was aspirated from individual animals for cytologic examination. This procedure was followed until the animals died (see table 4). In addition, enough ascitic fluid was pooled (table 3) on the second and third day after virus inoculation for titration in mice, as in experiment I. The resulting LD_{50} titers (last column, table 3) again indicated a very high rate of multiplication of the virus in the ascitic fluid, particularly if one considers the $10^{-7.50}$ titer obtained in mice that received an amount of virus equivalent to one intracerebral LD_{50}.

The results of cytologic observations summarized in table 4 indicate that the percentage of tumor cells in control smears obtained from noninfected mice varied from 65 to 87 percent, and the percentage of mitoses from 0 to 3 percent. Cells in karyorrhexis were absent, and degenerated cells and macrophages with phagocytized material were only observed once—on the third day after implantation of tumor.

In contrast to this, smears from mice injected with 10^{-4} dilution of virus showed destruction of tumor cells on the first day after inoculation. The oncolytic effect induced by 10^{-5}, 10^{-6} and 10^{-7} dilutions of virus was noted on the second day, and on the third day in mice inoculated with the highest dilution of virus.

Mitotic figures were observed in control animals in all three smears on the first day, in two of three smears on the second day, and in one of three smears on the third day. In those smears obtained from virus-injected animals mitoses were generally absent. However, in two instances, mitotic figures were noted in smears obtained from animals injected with 10^{-7} and 10^{-8} dilutions of virus. In these cases the appearance of mitotic figures preceded the onset of oncolysis. The presence of numerous degenerated and karyorrhectic cells preceded or coincided with the appearance of oncolysis.

Although there was no consistent selective response on the part of any type of non-neoplastic cell to oncolysis, in numerous smears from infected mice the polymorphonuclear leukocytes accounted for most of the nonmalignant cells. Histiocytes comprised 50 percent of the total cell count in one instance. In addition, 7 percent of macrophages containing phagocytic inclusions of broken-down nuclear fragments, illustrated in figure 2, were observed in mice injected with 10^{-8} dilutions of virus (third day). In general, the non-neoplastic cells presented no unusual features, except for a single instance when peculiar forms of histiocytes were noted. This will be described later under experiment IV and illustrated in figures 4, 5, and 6.

In experiment III, groups of five tumor-bearing mice were injected with

varying doses of Mengo virus, ranging from <1 (table 3) to 310,000 mouse LD_{50}. With the exception of mice injected with the highest (10^{-9}) dilution, all the animals succumbed to infection. Starting from the first day after virus inoculation, three mice in each group were tapped and smears of individual ascitic fluids were prepared for cytologic studies. In addition, as in the preceding experiment, ascitic fluids from a few animals (table 3) in each dilution group were pooled and titrated in mice. The resulting LD_{50} titers (table 3) were not as uniform as in the preceding experiments, which was probably due to the fact that in a few instances the amount of ascitic fluid obtained was so scanty that for titration purposes it had to be supplemented by the addition of peritoneal washings. This may account for the $<10^{-1.00}$ LD_{50} of fluid obtained from mice that received the equivalent of 310 intracerebral and 56 intraperitoneal LD_{50} of virus. Again, attention should be drawn to the high rate of multiplication of the virus in the ascitic fluid. For instance, in mice injected with 10^{-8} dilution, which thus received an equivalent of <1 intracerebral LD_{50} of virus, the infectious agent seemed to acquire a capacity to multiply 10 million times, as indicated by the resulting $10^{-7.50}$ LD_{50} titer of the ascitic fluid.

The cytologic findings are summarized in table 5. It may be seen that tumor cells in three noninfected control mice were numerous throughout the period of observation, with the exception of an unexplained and transient drop to 33 percent on the second day in the case of a single animal. No one type of non-neoplastic cell exceeded 24 percent in control smears. Mitoses varied from 0 to 4 percent, averaging 1.5 and 2 percent. Degenerated cells were either absent or represented at most 1 percent of the cellular element. Only a single cell in karyorrhexis was noted in the control smears. Not more than 2 percent of counted cells had phagocytic inclusions.

In infected animals destruction of tumor cells was induced even by small concentrations of virus in the inoculum (compare with table 3); however, the greater concentrations of virus caused an earlier manifestation of oncolysis. In few cases, as for instance in all mice injected with 10^{-7} dilution of virus and in individual animals injected with other dilutions, a decrease in the number of tumor cells was not observed prior to the death of the animal.

Mitotic divisions were not found in smears obtained from mice inoculated with 10^{-2} and 10^{-3} dilutions of virus. They were noted, however, following injection of mice with higher dilutions of virus.

Increase of degenerated cells and of cells in karyorrhexis followed infection of mice with a concentrated virus suspension. Figure 3 illustrates appearance of many cells in karyorrhexis as they were seen on the first day after inoculation of the 10^{-2} dilution of the virus. No definite increase of phagocytes was observed.

No selective response of histiocytes, polymorphonuclear leukocytes or lymphocytes to the decreased number of malignant cells was evident. In two animals the distinction between histiocytes and lymphocytes was

TABLE 5.—*Cytologic changes in the Ehrlich ascites tumor induced by Mengo virus—experiment III (in percentages of differential cell count for an individual mouse)*

Virus dilution inoculated	Mice tapped (days after virus inoculated)	Tumor cells			Histiocytes			Polymorphs			Lymphocytes			Mitoses			Degenerated cells			Cells in karyorrhexis			Cells with phagocytic inclusions		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
10^{-3}	1	61	1	X	13	12	....	5	78	....	1	8	.....	2	0	...	7	0	...	11	1	...	0	0	...
	2	45	2	....	9*	48	....	13	32	....	11	12	.....	0	0	...	19	4	...	2	2†	...	1	0	...
	3	X	X	....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10^{-3}	1	81	67	72	11	18	21	7	13	2	1	0	2	0	0	0	0	2	3	0	0	0	0	0	0
	2	1	0	X	31	36	....	17	26	....	51	36	.....	0	0	...	0	2	...	0	0	...	0	0	...
	3	X	X	....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10^{-4}	1	81	85	86	11	8	7	5	6	4	0	0	0	3	1	3	0	0	0	0	0	0	0	0	0
	2	48	79	85	26	7	6	9	2	1	8	1	1	0	0	0	5	7	1	3	4	5	1	0	1
	3	31	32	X	18	15	....	22	29	....	8	9	.....	0	0	...	16	9	...	5	5	...	0	1	...
	4	X	X	....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10^{-5}	1	92	82	79	2	7	16	4	6	3	0	1	2	2	2	0	0	0	0	0	0	0	0	2	0
	2	3	84	X	42*	10	....	16	3	....	34	1	.....	0	1	...	3	1	...	2	0	...	0	0	...
	3	0	X	....	74	.....	.....	11	.....	.....	12	.....	.....	0	...	...	1	.....	...	0	.....	.....	2	.....	.....
	4	X	....	....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10^{-6}	1	77	83	78	15	12	17	3	2	2	1	0	1	4	3	2	0	0	0	0	0	0	0	0	0
	2	74	5	13	11	50‡	9	11	5	9	2	40	67§	2	0	0	0	0	2	0	0	0	0	0	0
	3	58	19	0	10	37	43	15	9	6	15	33	51	2	2	0	0	0	0	0	0	0	0	0	0
	4	X	X	X	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10^{-7}	1	78	77	82	10	11	9	10	10	8	0	0	0	2	2	1	0	0	0	0	0	0	0	0	0
	2	77	80	86	13	10	9	3	7	1	3	2	0	1	1	2	3	0	1	0	0	1	0	0	0
	3	83	63	91	6	8	2	1	13	3	4	4	1	2	0	3	1	11	0	2	1	0	1	0	0
	4	X	X	X	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

10^{-8}	1	87	88	95	3	5	3	6	5	2	1	0	0	3	2	0	0	0	0	0	0	0	0	0	0
	2	70	75	88	11	11	7	12	12	2	6	1	2	1	1	1	0	0	0	0	0	0	0	0	0
	3	77	2	0	9	29	10	10	18	68	2	50	14	2	1	8	0	0	0	0	0	0	0	0	0
	4	X	X	X	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10^{-9}	1	81	87	88	8	4	4	4	6	3	2	2	0	5	1	5	0	0	0	0	0	0	0	0	0
	2	29	53	85	18	12	9	15	14	2	35	15	3	0	5	1	1	0	0	0	0	0	2	1	0
	3	60	22	46	10	19	20	13	20	20	15	39	13	2	0	1	+	0	0	0	0	0	0	0	0
	4	86	8	4	3	17	16	7	21	42	1	51	37	2	1	0	0	2	1	0	0	0	1	0	0
Control	1	94	72	78	6	18	11	0	6	7	0	0	0	0	1	3	0	1	1	0	0	0	0	2	0
	2	81	91	33	6	2	23	9	4	18	1	1	24	2	2	2	0	0	0	1	0	0	0	0	0
	3	57	83	76	9	10	12	12	5	9	20	1	3	2	1	1	0	0	0	0	0	0	0	0	0
	4	60	78	70	22	7	17	14	7	7	2	4	1	2	4	3	0	0	0	0	0	0	0	0	2

*Histiocytes and epithelioid cells.

†Nuclear debris present in smear but not included in counts of cells in karyorrhexis.

‡Distinction from immature polymorphonuclear made arbitrarily.

§Distinction from histiocytes made arbitrarily. I, II, III refer to individual mice. For explanation of other symbols, see table 4.

difficult; in a single animal that between histiocytes and immature polymorphonuclear leukocytes presented a problem. Presence of epithelioid histiocytes was noted in two mice on the second day after inoculation with 10^{-2} and 10^{-5} dilutions of virus, respectively.

In experiment IV viral inoculum consisted of serial tenfold dilutions ranging from 10^{-3} through 10^{-9}. Groups of six tumor-bearing mice were inoculated with each dilution and one group of six animals was held as noninfected control. The control mice and those injected with virus were tapped once every day, from the first day after virus inoculation until the death of the animal, enough fluid being aspirated from each individual mouse to prepare a smear. No attempt was made to determine the virus content in the ascitic fluids.

Results of the cytologic observations were again similar to those obtained in previous experiments. The details and the corresponding table are omitted for the sake of brevity of publication. It will merely be mentioned that large polygonal epithelioid elements, already observed in experiments II and III, were present in three mice on the fourth day after inoculation of virus (10^{-7} and 10^{-8} dilutions). Their finely vacuolated cytoplasm with phagocytic inclusions made it improbable that they might have been squamous cells introduced by an aspirating needle. Several examples of such uncommon histiocytes are illustrated in figures 4, 5, and 6. These cells were not observed in the previous study (*1*).

Discussion

The unique feature of the ascites tumor–virus system is that viruses come into contact with host cells susceptible to infection immediately after obtaining entry into the body of the animal. From the experiments performed thus far with 13 viral agents (*1*), the ascites cells have to be considered as nonsusceptible to infection with only three viruses: Semliki Forest, western and eastern equine encephalomyelitis. In addition to earlier observations (*1*), the comparative level of multiplications of the eastern virus in mice with tumors or toxoplasmosis indicated the "hostility" of the Ehrlich ascites tumor cells for the virus. The behavior of the remaining nine viruses (*1*) varied markedly from that observed with Semliki Forest, western and eastern viruses. The ascites cells were found to be "virus-susceptible" to this group, although the level of viral multiplication varied with the individual strain (*1*). The behavior of the Mengo virus indicates that the cell of the ascites tumor of mice seems to be the most favorable medium for the multiplication of this particular agent. The results of six comparative titration experiments with Mengo virus in mice confirm this observation. Viral infection was induced with particles of virus or aggregates of such particles that had failed to produce signs of illness when injected directly into the nervous tissue of the animal (table 3).

The high degree of susceptibility of the ascites tumor cells to infection with Mengo virus is intimately associated with their special vulnerability,

since the multiplying virus seemed to exert a destructive influence upon the host cells. Although it was impossible to carry out biologic assays on the effect of viral infection upon the neoplastic process (see above), the cytologic evidence accumulated in these experiments yields convincing data as to the marked oncolytic property of the virus. Some interesting features of oncolysis were noted in the daily observations of smears obtained from individual animals.

Destruction of tumor cells with the use of the more concentrated viral inocula was usually accompanied by disappearance of mitoses from corresponding smears. As a rule, this did not occur in mice in which oncolysis was induced by higher dilutions of virus. On the contrary, it was noted that in some cases the number of mitoses exceeded that observed in cells of noninfected ascitic fluid. Although abnormal mitotic figures were seen in malignant cells, normal mitoses were also observed in degenerated cells and it was uncertain whether the latter were of neoplastic or histiocytic origin. A more detailed investigation of the mitotic mechanisms involved is beyond the scope of this presentation.

Smears in which tumor cells were reduced in number usually showed a coincident simultaneous increase of cells in karyorrhexis. It may be important to note, however, that not all the cells in karyorrhexis were of the malignant variety.

Another observation concerns variations in the number of tumor cells encountered in smears of ascitic fluids from different animals inoculated with the same dilution of virus. In several mice, the last cell count failed to indicate a marked decrease in the number of tumor cells as the result of infection. A definite decrease in neoplastic cells was, however, found in other animals inoculated with the same dilution of virus. This might be explained by the assumption that a decrease of tumor cells could have occurred during the time elapsing between the last cell count and the death of the animal. It is also possible that some mice died of infection before oncolysis took place.[5]

In control animals variations in tumor cells were only minimal. Nevertheless, in two control mice, tumor cells were as low as 33 percent (experiment III, second day) and 25 percent (experiment IV, fifth day). The 33 percent cell count was transient and was followed by a high count on the following day. It might have been due to experimental error. The 25 percent of malignant cells in a control smear may possibly indicate that in rare instances exfoliation of tumor cells can be reduced even in untreated mice.

The unusually shaped histiocytes observed in this study were not seen in control smears of mouse ascites tumors. They resemble "epithelioid" cells present in sections of tissues from the granulomatous type of lesions. It appears likely that the transformation of histiocytes into "epithelioid"

[5] Supporting evidence for this hypothesis may be found in the results obtained with West Nile virus, which was found to interfere significantly with the progress of the neoplastic process, as indicated by the results of bioassay (*1*). However, cytologic criteria of oncolysis were lacking until experiments were performed in mice resistant to the lethal effect of the virus. Smears from these animals revealed that biologic evidence of oncolysis was supported by cytologic evidence (*3*).

cells may occur in the ascitic fluid of mice and that it results from ingestion of large quantities of phagocytized material.

Some justification may be necessary for the detailed tabulation of various types of non-neoplastic cells. This was done in order to detect any selective responses to the destruction of malignant cells that might have occurred on behalf of one of the three major groups of non-neoplastic cells. Although such responses proved to be inconsistent in the present study, a selective lymphocytic response was observed in a previously conducted investigation of the exfoliated cells in pleural and peritoneal fluids of inoperable cancer patients subjected to induced infection with West Nile virus (*4*). Conversely, however, the evaluation of cellular changes induced by viral infection and studied by the differential cell-count method appeared to be much more accurate in the case of mouse ascites tumors than in that of human serous effusions. This is partially due to the possibility of better distinguishing different cellular types in ascitic fluids of mice than in the case of pleural and peritoneal fluids from human subjects.

Summary

The infectivity of the Mengo encephalitis virus for the Ehrlich ascites tumor of mice has been investigated, and it was found that the virus multiplies at a faster rate in the ascites tumor than in mouse brain. Invasion of the tumor by the virus is followed by marked cytologic changes which lead ultimately to the destruction of the tumor cells. These phenomena are briefly discussed.

Literature Cited

(*1*) Koprowska, I., and Koprowski, H.: Morphologic and biologic changes in a mouse ascites tumor following induced infection with certain viruses. Cancer Research 13: 651–660, 1953.

(*2*) Love, R., Koprowski, H., and Cox, H. R.: Cytological studies on the Ehrlich ascites tumor before and after infection with Bunyamwera virus. Cancer Research 13: 350–357, 1953.

(*3*) Koprowski, H., and Love, R.: The effect of virus infections upon ascites tumors of mice. (Abstract.) Proc. Am. Assoc. Cancer Research 1: 30, 1953.

(*4*) Koprowska, I.: Preliminary observations on the morphologic changes of exfoliated cells in exudates of cancer patients following induced viral infections. Am. J. Path. 29: No. 5 (in press), 1953.

PLATE

PLATE 48

(*All photomicrographs are* × *600*)

FIGURE 1.—Ehrlich ascites tumor cells in a control smear.

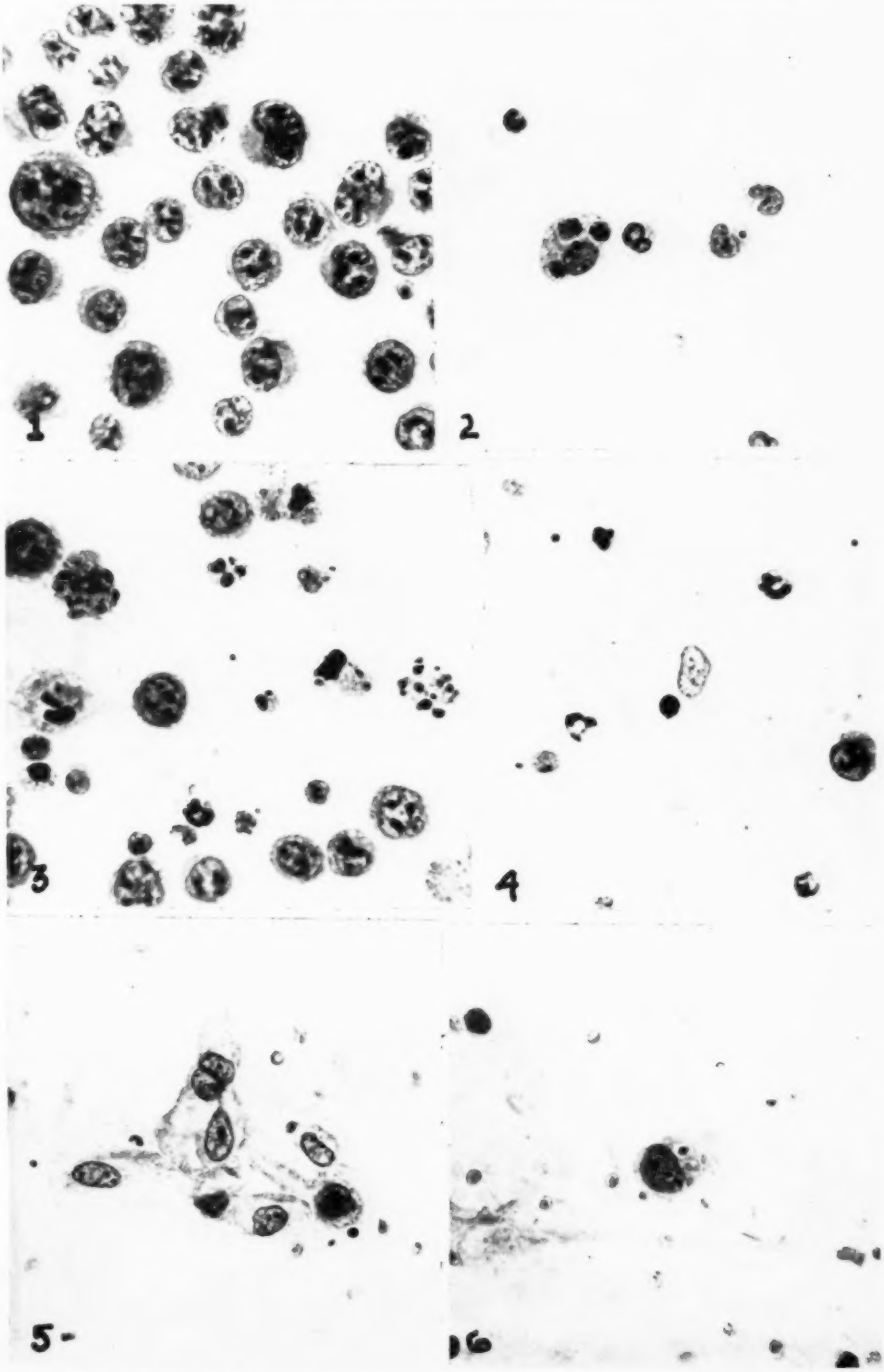
FIGURE 2.—Histiocytes containing phagocytic inclusions on the third day following inoculation of 10^{-6} dilution of virus (experiment II).

FIGURE 3.—Numerous cells in karyorrhexis on the first day after inoculation of 10^{-2} dilution of virus (experiment III).

FIGURE 4.—A large epithelioid histiocyte found on the second day after inoculation of 10^{-2} dilution of virus (experiment III).

FIGURE 5.—A group of large epithelioid histiocytes seen on the third day after inoculation of 10^{-7} dilution of virus (experiment II).

FIGURE 6.—Unusual histiocyte similar to those illustrated in figures 4 and 5, and containing cytoplasmic inclusions that favor the theory of its phagocytic nature (experiment IV).



273201—53——20

Damage Induced in Sarcoma 37 with Chemical Agents. VII. Diphenylpropylamines, Diphenylpropylenediamines, Diphenylalkanes, and Related Compounds [1]

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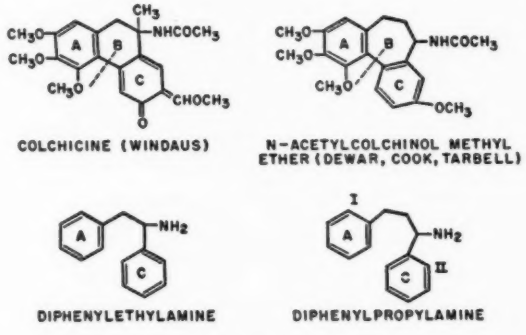
Following the discovery of the powerful action of colchicine as a mitotic poison, chemists began to synthesize analogs to see whether this property would be retained in related compounds of simpler structure (*1–4*). On the basis of the structure for colchicine proposed by Windaus (*5*), *i.e.* a reduced phenanthrene ring system, Lettré and Fernholz (*2*) prepared a series of derivatives of diphenylethylamine, a type of compound which would be formed were the B ring of colchicine to be opened appropriately (text-fig. 1). A considerable number of such compounds were found (by Lettré and co-workers) to inhibit mitosis of fibroblasts in tissue culture.[3] The results of his systematic and extensive investigations have recently been summarized by Lettré (*6*). In this Laboratory we have investigated a large number of these compounds and found a large proportion of them to be potent in damaging Sarcoma 37 (*7*). These will be reported in detail in a separate communication.

Later chemical evidence indicated that the Windaus formula for colchicine was in error, and it was demonstrated that both the B and C



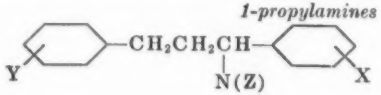
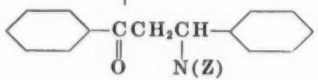
Text-figure 1.

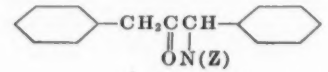
[1] Received for publication August 7, 1953.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

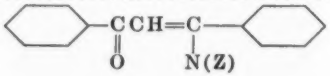
[3] Unless otherwise stated, effects reported by Lettré *et al.* are for this tissue culture technique.

TABLE 1.—*Damage induced in Sarcoma 37 with a single injection of derivatives of 1,3-diphenylpropylamine*

NCI No.	Compound			Source	Dose* (μg./gm.)	Mortality†	Damage induced in Sarcoma 37‡
	1-propylamines 						
	X	Y	Z§				
1878	*p*-OCH_3	2′,3′-dimethoxy	H	E	60	0/15	0/15
1879	*p*-OCH_3	4′-methoxy	H	E	35	2/15	0/13
2052		3′,4′-dimethoxy	H	E	300	4/30	22/26
					250	0/8	0/8
2053	*p*-OCH_3	3′,4′,5′-trimethoxy	H	E	450	1/14	7/13
					400	0/22	0/22
2559	3,4,5-tri OCH_3	3′,4′-methylenedioxy	H	E	200	1/16	5/7
					100	0/8	2/8
2583		3′,4′-methylenedioxy	H	E	80	0/24	9/24
					40	0/8	2/8
2910	*m*-OCH_3	3′,4′,5′-trimethoxy	H	E	250	0/16	8/16
					150	0/16	6/16
3122	*m*-OH	3′,4′,5′-trimethoxy	H	E	700	2/16	5/14
					500	0/8	1/8
3123	*m*-OCH_3	3′,4′,5′-trimethoxy	Acetyl	E	1,000‖	0/16	1/16
3125	*p*-OCH_3	3′,4′,5′-trimethoxy	Acetyl	E	1,000‖	0/8	0/8
2564			*p*-diethylaminophenyl	B	500‖	0/8	0/8
							
2576			benzyl	B	1,000‖	0/8	0/8
1082			piperidino	B	1,000‖	1/32	2/31
579			morpholino	A	1,000‖	0/8	0/8

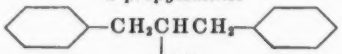


1805	morpholino	D	800	5/24	6/19
			600	0/8	0/8
2067	di-isopropanol	D	150	1/8	3/7
			125	0/16	5/16
2540	bis-(β-methoxyethyl)	D	800	1/16	2/15
2548	bis-(β-hydroxyethyl)	D	200	5/8	1/3
			150	0/16	2/16

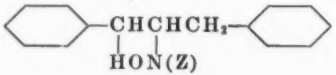


591	morpholino	A	1,000‖	0/8	0/8

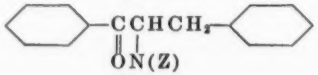
2-propylamines



470	β-hydroxyethyl	F	30	0/8	0/8
2292	bis-(β-hydroxyethyl)	D	400	6/16	10/10
			300	3/8	2/5



587	morpholino	A	600	0/16	0/16
			500	0/8	3/8
2563	piperidino	B	400	0/8	5/8
			350	1/8	3/7



578	morpholino	A	1,000	0/8	0/8
1299	piperidino	B	1,000	0/16	0/16
2562	β-hydroxyethyl	B	400	2/8	4/6
			300	0/8	2/8
2646	diallyl	D	600	3/17	4/14

*Aqueous vehicle used unless otherwise indicated.

†Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice injected}}$.

‡Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated control mice did not show induced damage.

§In primary amines N(Z) = NH_2.
In secondary amines N(Z) = NHR.
In tertiary amines N(Z) = $N(R)_2$ or a heterocyclic ring (piperidine or morpholine).

‖Olive-oil vehicle.

rings of colchicine were seven-membered (*8–12*). Negative results have been reported by Lettré and co-workers (*2–4*) and by Skipper *et al.* (*13*) with a small number of diphenylpropylamines, the analogous type of compounds that would be formed if the seven-membered B ring of colchinol methyl ether (an aromatized derivative of colchicine) were split (text-fig. 1). We also examined a series of this type of compound, but found that some of them possessed activity against Sarcoma 37. In addition, 34 diphenylpropylenediamines and 26 diphenylalkanes, in which the length of the aliphatic chain was varied from 3 to 7, were examined. The results of these experiments are presented here.

Materials and Methods

The sources of the compounds employed are given below. For each compound in the tables the source is indicated by one of the following code letters.

Code	*Source*
A	Dr. Lyndon F. Small, National Institutes of Health, Bethesda, Md.
B	Dr. Robert E. Lutz, University of Virginia, Charlottesville, Va.
C	Chemical-Biological Coordination Center, National Research Council, Washington, D. C.
D	Dr. L. H. Goodson, Midwest Research Institute, Kansas City, Mo.
E	Dr. R. B. Sandin, University of Alberta, Edmonton, Alberta
F	George A. Breon and Co., Kansas City, Mo.
G	B. F. Goodrich Co., Brecksville, Ohio
H	Hoffman-LaRoche, Inc., Nutley, N. J.
I	Dr. L. L. Engel, Massachusetts General Hospital, Boston, Mass.
J	Dr. Erich Mosettig, National Institutes of Health, Bethesda, Md.

The methods employed in evaluating the damage induced in Sarcoma 37 were described previously (*14, 15*). The observations, in most cases, were made 24 hours after a single subcutaneous injection in mice bearing 6-day-old implants of Sarcoma 37. In some instances, observations were also made after 8 and 48 hours; such data have been combined to simplify the tabulation. For groups of compounds yielding negative results, only the highest dose used is reported, together with the mortality during the observation period (usually 24 hours).

Results

Table 1 summarizes the results obtained following injection of 1,3-diphenylpropylamines. The compounds have been grouped chemically in accordance with the position of the amino group on the propane chain (1-propylamines and 2-propylamines) and with the degree of oxidation of the chain (alcoholic or ketonic substituents). In the case of the 1-propylamines the variations in structure were a result of different substituents on the phenyl rings (A and B), but with the 2-propylamines the variations were in substituents on the amino group. In many instances, the amino group formed part of a heterocyclic ring (morpholine or piperidine).

Of the 27 derivatives of 1,3-diphenylpropylamine examined, 17 showed activity in damaging Sarcoma 37, but a dose near the maximum tolerated (MTD) was required. In several instances only a few of the tumors in a group were damaged. Of the 10 inactive compounds of this group, 5 were insoluble in aqueous medium and were consequently injected as a suspension in oil. Of special interest are NCI-2910 and 3122, which are the diphenylpropylamine compounds that would result if the B-ring in colchinol methyl ether and colchinol, respectively, were opened as shown in text-figure 1. Both these amines induced damage in the tumors. On the other hand, NCI-3123 is the analogous compound that would result by opening the B-ring of N-acetylcolchinol methyl ether. This amine yielded essentially negative results; it was, however, insoluble in aqueous vehicle.

Table 2 lists the effects obtained with 2,3-diphenylpropylamines. Four of the 10 compounds in this series showed activity in damaging Sarcoma 37.

Table 3 lists 34 diphenylpropylenediamines, none of which showed potency in damaging Sarcoma 37; the table gives the highest dose used and the mortality observed. Both of the amino groups in this series of

TABLE 2.—*Damage induced in Sarcoma 37 with a single injection of derivatives of 2,3-diphenylpropylamine*

NCI No.	Compound (*in aqueous vehicle*)	Source	Dose (*μg./gm.*)	Mortality*	Damage induced in Sarcoma 37†
99v	N - Acetyl -β - (4 - methoxyphenyl)-γ-(3, 4, 5-trimethoxyphenyl) - *n* - propylamine	I	1000	0/8	0/8
2593	1, 2-Dipheny-3-piperidylpropanol - 1·HCl	B	300	2/8	2/6
			200	0/16	2/16
581	1, 2 - Diphenyl - 2 - (N - morpholinomethyl)-ethanone·HBr	A	500	0/8	0/8
743	α-Dimethylaminomethyl-α-phenyl - *p*-methoxyacetophenone·HBr	H	150	0/8	0/8
83x	α - Dimethylaminomethyl - α-(*p*-tolyl)-acetophenone·HCl	H	200	0/15	0/15
96v	α-Dimethylaminomethyl-α-(3,4-xylyl)-acetophenone·HCl	H	250	0/8	0/8
1002	1,2-Diphenyl-2-(N-piperidinomethyl)-ethanone	B	350	2/16	4/14
1159	α-4-Piperidylmethylbenzoin	B	1000	0/8	0/8
1306	α - Dimethylaminomethyl - α - phenyl-acetophenone·HCl	H	150	2/8	2/6
			125	0/8	2/8
1307	α-Dimethylaminomethyl - *p*-methoxy-α-(*p*-methoxyphenyl) - acetophenone ·HCl	H	175	3/16	10/13
			150	1/8	6/7

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice injected}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated control mice did not show induced damage.

compounds were incorporated in heterocyclic rings. Most of them were insoluble in aqueous vehicle.

A group of 26 diphenylalkanes, with a chain length varying from 3 to 7 but without a substituted amino group on the chain, were examined. These were found to be inactive in producing damage in the tumors. Table 4 lists the compounds, with the same type of information as in table 3.

TABLE 3.—*Diphenylpropylenediamines which did not induce necrosis in Sarcoma 37 following a single subcutaneous injection*

NCI No.	Compound* (*in aqueous medium*)	Source	Dose (*μg./gm.*)	Mortality†
	Dipiperidino- compounds			
55	α,β-Dipiperidinobenzylacetophenone	A	1000	0/7
56	2,3-Dipiperidino-1-anisyl-3-phenylpropanone-1	A	1000	0/5
70	1-(4-Phenoxyphenyl)-2,3-dipiperidino-3-phenyl-propanone-1	A	1000	0/8
	2,3-Dimorpholino-propanone-1			
57	1-(2,5-Diphenylfuryl-3)-3-phenyl	A	1000	0/8
58	1-(2,4-Di-isopropylphenyl)-3-phenyl-	A	1000	0/8
59	1-(2,5-Dimethylphenyl)-3-phenyl-	A	250	0/8
6v	1-(4-Isopropyl)-3-(4-methoxyphenyl)-	A	1000	0/11
6x	1-Phenyl-3-(3-methylphenyl)-‡	A	1000	0/8
60	1-Phenyl-3-(4-isopropylphenyl)-	A	1000	0/8
61	1-(4-Phenoxyphenyl)-3-phenyl-	A	1000	0/8
71	1-(1-Naphthyl)-3-phenyl-	A	1000	0/8
72	1-*p*-Bibenzyl-3-phenyl-	A	1000	0/8
73	1-*p*-Bromophenyl-3-phenyl-	A	1000	0/8
74	1-(4-*tert.*-Butylphenyl)-3-phenyl-	A	1000	0/8
75	1-(4-Propoxyphenyl)-3-phenyl-	A	1000	0/8
76	1-(2-Naphthyl)-3-phenyl-	A	1000	0/8
78	1,3-Di-(4-methoxyphenyl)-	A	1000	0/15
8x	1-(3-Methyl-4-chlorophenyl)-3-phenyl-	A	1000	0/8
80	1-Phenyl-3-(4-chlorophenyl)-	A	300	0/10
534	1-Phenyl-3-(*o*-methoxyphenyl)-	J	1000	0/24
539	1,3-Diphenyl-	J	1000	0/8
54x	1-Anisyl-3-phenyl-‡	J	1000	0/6
540	1-*p*-Tolyl-3-phenyl-	J	1000	0/8
541	1-(4-Isopropylphenyl)-3-phenyl-	J	1000	0/8
542	1-(4-Butoxyphenyl)-3-phenyl-	J	1000	0/8
543	1-(4-Isopropoxyphenyl)-3-phenyl-‡	J	1000	0/8
	Miscellaneous			
54	α,β-Di-N-morpholinobenzylacetone‡	A	150	0/8
62	1-Anisyl-3-phenyl-2,3-di-(1,2,3,4-tetrahydroisoquinolino)-propanone-1	A	1000	0/8
77	1,3-Diphenyl-2-piperidino-3-morpholinopropanone-1	A	150	0/8
79	2,4-Diphenyl-3,4-dimorpholinobutanol-2‡	A	500	0/8
8v	1,3-Diphenyl-2-morpholino-3-anilinopropanone-1	A	1000	0/8
537	4-(4-Chlorophenyl)-2,3-di-(N-morpholino)-butanone-2	J	250	0/8
54v	α,β-Dimorpholinobenzylpinacolone‡	J	1000	0/8
599	1,3-Diphenyl-2,3-di-(N-methyl-benzylamino)-propanone-1	J	1000	0/8

*Most of these compounds were made available to us from the collection of compounds at the National Institutes of Health that were used in a survey of compounds for antimalarial activity.

†Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice injected}}$.

‡In olive-oil vehicle.

TABLE 4.—*Diphenylalkanes which did not induce necrosis in Sarcoma 37 following a single subcutaneous injection*

NCI No.	Compound (*in olive-oil vehicle*)	Source	Dose (*μg./gm.*)	Mortality*
	Diphenylpropanes			
1292	Dimethyldi-(*p*-hydroxyphenyl)-cyclobutane	G	1000	0/8
1566	γ-Nitro-β-phenylbutyrophenone	C	1000	0/8
1567	β-Benzoyl-β-bromo-α-phenylpropionic acid methyl ester	C	1000	0/8
1569	Tetraphenylallene	C	1000	0/8
1571	1,3-Diphenyl-1,3-propanedione	C	1000	0/8
1576	2-Bromo-1,3-diphenyl-1,3-propanedione	C	700	1/8
1577	α,β,4-Tribromo-β-phenylpropiophenone	C	1000	0/8
1578	α,β-Dibromo-β-phenylpropiophenone	C	1000	0/8
1579	β-*p*-Chlorobenzoyl-α-phenylpropionic acid methyl ester	C	1000	0/8
1580	*p*-Bromo-α-hydroxymercuri-β-methoxy-β-phenylpropiophenone acetate	C	175	0/8
1582	α-Chloro-α,β-diphenylpropiophenone	C	1000	0/5
1585	2-Nitro-3-phenyl-1-cyclopropylphenylketone	C	1000	0/7
1586	γ-Nitro-β-(*m*-nitrophenyl)-valerophenone	C	1000	0/7
1587	γ-Nitro-β-phenylvalerophenone	C	1000	0/7
1592	γ-(*p*-Chlorophenyl)-γ-oxo-α-phenylbutyronitrile	C	1000	0/8
1593	2-Nitro-3-phenyl-1-cyclopropyl-*p*-chlorophenyl ketone	C	1000	0/8
1594	α-Bromo-γ-nitro-β-(*p*-nitrophenyl)-butyrophenone	C	1000	0/8
1595	γ-Bromo-γ-nitro-β-phenylbutyrophenone	C	1000	0/8
1597	γ-Bromo-*p*-methoxy-γ-nitro-β,γ-diphenylbutyrophenone	C	1000	0/8
1601	β-Bromo-β-(*p*-chlorobenzoyl)-α-phenylpropionic acid methyl ester	C	1000	0/8
1602	*p*-Chloro-γ-nitro-β-phenylbutyrophenone	C	1000	0/8
	Diphenylbutanes			
702	1,4-Di (*p*-chlorophenyl)-2-N-piperidino-butanedione-1,4	B	1000	0/8
1588	β-Benzoyl-β-nitro-β′-phenylisobutyric acid methyl ester	C	1000	0/8
1596	β-Bromo-β-benzoyl-β′-nitro-β′-phenylisobutyric acid†	C	1000	0/8
	Diphenylpentanes			
1575	α-Benzohydryl-γ,γ-dibenzoyl-β-phenylbutyrophenone	C	1000	0/8
	Diphenylheptanes			
1589	α,α-bis(2-Benzoyl-1-phenylethyl)-α-tolunitrile	C	1000	0/8

* Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice injected}}$.

† In aqueous vehicle.

Discussion

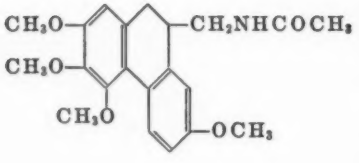
As with the biphenyl and tropolone derivatives described in a previous publication (*16*), high potency in damaging Sarcoma 37 was not exhibited by the diphenylpropylamines. These simpler analogs of colchicine did not yield any compounds with as high a potency, or with as great a range between the maximum tolerated dose (MTD) and the minimum effective dose (MED), as shown by colchicine and some of its derivatives. Nevertheless, as with the tropolones, it is of interest that some potency in damaging Sarcoma 37 was retained by some of the diphenylpropylamines. It might be worth while to investigate compounds analogous to a simplified

colchicine molecule in which a seven-membered tropolone ring replaced one of the two phenyl groups in the diphenylpropylamines. Although the diphenylpropyl compounds are analogous to colchinol derivatives, which have shown high potency in our experiments (*15*), it is conceivable that the presence of these two functional groupings (propylamine and tropolone) of the colchicine molecule might result in greatly enhanced potency.

The diphenylpropylamines appear to differ from the colchicines (*15*) in the apparent relationship between potency and solubility in aqueous vehicle. The majority of the insoluble 1,3-diphenyl compounds were inactive or showed negligible potency. Of the five water-soluble derivatives that were inactive, doses were generally less than lethal, inasmuch as deaths were obtained with only one compound at the doses administered; it is possible that higher doses of these compounds might have induced damage.

Activity was shown by five of the eight 2-amino-1,3-diphenylpropylamines. The position of the amino group in these compounds is not analogous to that in the colchicine molecule. This raises the question as to whether a similar displacement of the amino group in colchicine would yield compounds that were potent. So far we are not aware of any such compounds. Šantavý (*17*) has recently published data indicating that one of the compounds (compound F) he had isolated from the colchicum plant may be isomeric with trimethylcolchicinic acid methyl ether, but with the amino group shifted to the position analogous to that in the 2-propylamines. The biologic activity of this newly found component of the colchicum plant should be interesting to investigate. Kenner (*18*) and Cook *et al.* (*19*) have also prepared a dibenzcycloheptane derivative with an amino group in a position analogous to the 2-propylamines, but no information is available as to its biologic properties.

Although the 2,3-diphenylpropylamines are not strictly analogous either to the older Windaus formulation of colchicine or to the newer Dewar-Cook-Tarbell one, interest in this type of structure arose from the report by Cook and Engel (*1*) that Brues [personal communication in (*1*)] had found that this type of compound had an effect on cells. In addition, Cook *et al.* (*20*) prepared the analogous phenanthrene derivative (below) which Lettré and Hartwig (*4*) found active in inhibiting mitosis of normal fibroblasts in cultures. Lettré and co-workers (*2*, *21*) found these propylamines (including the amine that would occur if the middle ring of this phenanthrene derivative were opened appropriately) to be inactive. In our experiments the analogous acetylated amine (NCI-99v, table 2), was also inactive. Nevertheless, we were able to induce tumor damage with 4 propylamines of this type. These active compounds all had the propane chain in some stage of oxidation (alcoholic or ketonic) and the amino group was a tertiary one.



For several years Lettré and co-workers (*2, 4, 21, 22*) attempted to correlate the biologic activity of opened ring structures (diphenylethylamines and diphenylpropylamines) with their closed ring analogs (phenanthrene, colchicines, colchinols, etc.). The diphenylethylamines were active in inhibiting mitosis, whereas their phenanthrene analogs were inactive. Obversely, the diphenylpropylamines were inactive in their hands, whereas the closed ring analogs related to colchicine were active. Since they were unable to obtain any good or consistent correlation between open and closed ring analogs, Lettré and Hartwig (*4*) concluded that no biologic analogy existed, and that the diphenylethylamines should be considered a specific, independent type of configuration for antimitotic activity. They considered this configuration to be unrelated to the colchicine-type structure.

It is not possible for us to draw a similarly positive conclusion between the tumor-damaging potency and structure of colchicine and of the diphenylaminoalkanes as Lettré *et al.* have drawn for inhibition of mitosis of fibroblasts. The tumor-damaging potency we have observed with both types of propylamines is not inconsistent with tumor-damaging activity of their closed-ring analogs, colchinol and its related derivatives. Similarly the antimitotic activity or tumor-damaging potency of γ-substituted tropolones observed by Katsura *et al.* (*23–25*) and by us (*16*) is not inconsistent with the activity of their analog, colchicine. Apparently none of these structural analogs account for the high potency exhibited by the colchicine-type configuration.

Correlations between chemical structure and biologic activity may vary, depending on the particular biologic phenomenon being observed. There is no reason to expect that different phenomena (antimitotic activity, tumor-necrotizing activity, etc.) observed by Lettré and by us should necessarily parallel each other as chemical analogs of compounds are evaluated. As a matter of fact, even with simple derivatives of colchicine there have been quantitative variations between observations of these two phenomena with the same compounds. It would not be unusual to expect even qualitative differences to occur, once radical changes in structure are effected, as is the case with diphenylpropylamines.

Summary

1) Seventeen of 27 derivatives of 1,3-diphenylpropylamine induced damage in Sarcoma 37 following a single subcutaneous injection.

2) Four of 10 derivatives of 2,3-diphenylpropylamine induced damage in the tumors.

3) Thirty-four diphenylpropylenediamines and 26 diphenylalkanes, with carbon chains varying from 3 to 7 in length, were inactive in producing tumor damage.

References

(*1*) Cook, J. W., and Engel, L. L.: Colchicine and related compounds. Part II. Synthesis of a simple analogue of N-acetylcolchinol methyl ether. J. Chem. Soc. 198–200, 1940.

(2) LETTRÉ, H., and FERNHOLZ, H.: Beitrag zur Beziehung zwischen der Mitosegiftwirkung und der Konstitution von Colchicinderivaten. Ztschr. physiol. Chem. 278: 175–200, 1943.

(3) LETTRÉ, H.: Zur Chemie und Biologie der Mitosegifte. Angewandte Chem. 63: 421–430, 1951.

(4) LETTRÉ, H., and HARTWIG, E.: Vergleich ringgeschlossener und ringoffener Verbindungen vom Colchicintyp auf ihre antimitotische Wirkung. Ztschr. physiol. Chem. 291: 164–168, 1952.

(5) WINDAUS, A.: Untersuchungen über die Konstitution des Colchicins. Liebigs Ann. 439: 59–75, 1924.

(6) LETTRÉ, H.: Some investigations on cell behavior under various conditions: A review. Cancer Research 12: 847–860, 1952.

(7) DOWNING, V., HARTWELL, J. L., LEITER, J., and SHEAR, M. J.: Effect of a single injection of colchicine, colchicine derivatives and related compounds on mouse tumors. (Abstract.) Cancer Research 9: 598, 1949.

(8) DEWAR, M. J. S.: Structure of colchicine. Nature, London 155: 141–142, 1945.

(9) COHEN, A., COOK, J. W., and ROE, E. M. F.: Colchicine and related compounds. I. Some observations on the structure of colchicine. J. Chem. Soc. 194–197, 1940.

(10) BARTON, N., COOK, J. W., and LOUDON, J. D.: Colchicine and related compounds. Part V. The structure of Windaus' deaminocolchinol methyl ether. J. Chem. Soc. 176–180, 1945.

(11) ARNSTEIN, H. R. V., TARBELL, D. S., Huang, H. T., and SCOTT, G. P.: The structure of ring C of colchicine. J. Am. Chem. Soc. 70: 1669, 1948.

(12) RAPOPORT, H., WILLIAMS, A. R., and CISNEY, M. E.: The synthesis of *dl*-colchinol methyl ether. J. Am. Chem. Soc. 72: 3324–3325, 1950.

(13) SKIPPER, H. E., BELL, M., and CHAPMAN, J. B.: Screening of a diverse series of candidate antileukemic agents. Cancer. 4: 360–362, 1951.

(14) LEITER, J., DOWNING, V., HARTWELL, J. L., and SHEAR, M. J.: Damage induced in Sarcoma 37 with podophyllin, podophyllotoxin, *alpha*-peltatin, *beta*-peltatin and quercetin. J. Nat. Cancer Inst. 10: 1273–1293, 1950.

(15) ———: Damage induced in Sarcoma 37 with chemical agents. III. Colchicine derivatives related to trimethylcolchicinic acid and to colchinol. J. Nat. Cancer Inst. 13: 379–392, 1952.

(16) LEITER, J., HARTWELL, J. L., KAHLER, J. S., KLINE, I., and SHEAR, M. J.: Damage induced in Sarcoma 37 with chemical agents. VI. Biphenyl, fluorene, phenanthrene, and tropolone derivatives. J. Nat. Cancer Inst. 14: 365–374 1953.

(17) ŠANTAVÝ, F.: Látky ocúnu a jejich deriváty XXVII. Příspěvak ke konstituci látky F. Chemike listy 46: 368–373, 1952.

(18) KENNER, J.: The formation of cyclic compounds from derivatives of 2:2′-ditolyl. J. Chem. Soc. 103, Part 1: 613–627, 1911.

(19) COOK, J. W., DICKSON, G. T., ELLIS, D., and LOUDON, J. D.: Colchicine and related compounds. Part VI. 3:4:5:6-dibenzycyclohepta-1:3:5-triene. J. Chem. Soc. 746–750, 1947.

(20) ———: Colchicine and related compounds. Part VII. J. Chem. Soc. 1074–1078, 1949.

(21) LETTRÉ, H., HAEDE, W., and SCHÄFER, L.: Zur Mitosegiftwirkung substitutierte α-Phenylzimtsäurenitril. Ztschr. physiol. Chem. 289: 298–309, 1952.

(22) LETTRÉ, H., ALBRECHT, M., and FERNHOLZ, H.: Ein einfaches Mitosegift. Naturwiss. 29: 390, 1941.

(23) KATSURA, S., SATO, K., AKAISHI, K., NOZOE, T., and KITAHARA, Y.: Effect of tropolone and related compounds on Yoshida Sarcoma. I. Proc. Jap. Acad. 27: 31–35, 1951.

(24) ———: Effect of tropolone and related compounds on Yoshida Sarcoma. II. *Ibid* 27: 36–38, 1951.

(25) ———: Effect of tropolone and related compounds on Yoshida Sarcoma. III. *Ibid* 27: 250–254, 1951.

The Glycogen Content of Cell Suspensions Prepared from Massive Tissue Culture: Comparison of Cells Derived from Mouse Connective Tissue and Mouse Liver [1]

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In view of the limited knowledge concerning the character of cells maintained for extended periods *in vitro* and since it is exceedingly difficult or impossible, on the basis of morphology or growth characteristics, to identify cells that have been maintained for years through subculture as cell suspensions, it has been deemed highly desirable to obtain information on a normally occurring specialized function.

Homogenates from normal liver are reputed to inactivate the hormone estradiol *in vitro*, and the failure of homogenates from strain 721 liver cells from the mouse to inactivate estradiol (*1*) raised grave questions as to the identity of the cells. Accordingly it was decided to examine another function, that of glycogen storage, to see if the cells still possessed this faculty. At the same time a study was to be made of the pure strain L fibroblasts from the mouse (*2*), which were grown under the same conditions, in order to have a comparison of glycogen content. This study was predicated on the hypothesis that in the liver of the animal the parenchymal cells are the primary site for storage of glycogen, and that if glycogen storage occurred under *in vitro* conditions as in the present study it would be greater in liver parenchymal cells than in similarly cultured connective tissue fibroblasts.

Should it be found that the strain 721 cells were able to elaborate a greater concentration of glycogen than the fibroblasts, it would be strong supportive evidence for their identity as liver epithelium. If it were found that the two strains had the same glycogen content or that there was no glycogen in either, the following alternatives would be presented: the strain 721 cells did not actually originate from liver epithelium; the strain 721 cells had been so altered by long maintenance in culture as to lose any specialized capacity to store glycogen; the culture medium, although exceedingly effective in promoting rapid multiplication of the cells, was

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lacking or too low with respect to some factor or factors needed for glycogen formation and storage in the intact animal; or finally, that there were toxic materials formed in the used medium blocking glycogen storage.

The primary result of this study was that under the conditions maintained, the strain 721 cells believed to be liver epithelium had a glycogen content some 25 times as great as that of the strain L (929) mouse fibroblasts. Concurrently, studies made on the rate of disappearance of glucose from the medium in which the cells were growing, presumably thus measuring rate of utilization of the glucose by the cells, revealed that this rate was very similar for the two cell types despite the greater glycogen content of the liver cells.

Experimental Procedure

1) Design of experiments.—Two designs of experimental conditions were used: the first was set up chiefly for a comparison of glycogen content of the two cell types in massive culture; the second for a study of glucose utilization from the medium as well as comparison of glycogen content.

2) Validation of procedure for glycogen.—It was necessary to adapt the excellent procedure of Good, Kramer, and Somogyi (*3*) to the manipulative procedures made necessary by flask culture of the cells and to the microgram quantities of glycogen expected from the flask culture; it was also necessary to ascertain the limits of reliability of the procedure when applied to these minute amounts. Accordingly considerable time was spent in establishing these limits, and the results of this examination are briefly reviewed. Nelson's (*4*) sugar and color reagents were used for estimation of the reducing material after hydrolysis of the glycogen except that the amount of sulfate added to the former reagent was reduced by 10 percent. Otherwise there was trouble with the nearly saturated sulfate solution giving crystals.

The cells were packed in the centrifuge tube at 1,500 × g, washed once with saline, the volume of the cells was ascertained, and alkali added as required. In view of the minute amounts of glycogen found in the strain L fibroblasts it was necessary to exercise considerable attention to detail in the procedure, since any reducing material such as aldehyde from the alcohol precipitation of the glycogen reacted to give high results. Samples were ordinarily run in duplicate, so that starting with about one gram of material, in an instance such as that of strain 929 cells of date 7/14/50 from table 2, the final spectrophotometry was done on a solution containing about 5 micrograms as glucose. The horse serum–embryo extract medium gave a barely detectable value of 2 micrograms per ml. of apparent glycogen when put through the procedure.[5] Because of the saline wash it appeared unnecessary to subtract such a figure, because when the milliliter of medium was decanted and the tube then washed with saline as was

[5] Since this work was completed a report has appeared by Leibson (*5*) [*cf.* Lee (*6*)], wherein he finds the liver of the 9-day chick embryo to contain 500 milligrams percent glycogen. It seems very probable that this may be the primary source of glycogen of the medium as here used.

done with the cells, the resulting end value was indistinguishable from the reagent blank.

Glycogen was added to the medium in concentrations simulating those obtained from the cells of the cultures, and analyses were carried out to see what quantities could be recovered. Several experiments were made in the low range of 3 to 12 micrograms of glycogen per flask, to cover the general range of glycogen found in the fibroblasts and delimit the reliability of the analyses for the strain 929 fibroblasts. In twelve such experiments where the average quantity added was 7.7 micrograms, recovery was 7.1 micrograms. In nine similar experiments where added amounts ranged from 21 to 90 micrograms per flask to simulate the quantities found with the strain 721 liver epithelial cells, the average quantity was 40 micrograms added, and the average for the quantity recovered was 39.9 micrograms. The slightly better recovery with the larger quantity is probably ascribable in part to the greater facility in sedimenting and collecting the precipitate of glycogen. Theoretically recovery of added material should be slightly low in all instances. The fact that a few recoveries are high must reflect the difficulties of removing all extraneous reducing material. Since the sample of 721 cells having the lowest glycogen is still 20 times as high in glycogen as the lowest of the 929 series, there was adequate material to run a preliminary estimation and then make suitable volume adjustments to bring the final estimation into the optimum range of 20 to 50 micrograms.

Replicate analyses of the strain 721 cells from one experiment are shown in table 1, inasmuch as an experiment of this sort more nearly reflects the experimental conditions encountered when dealing with the material under study as contrasted with the experiments on recovery of known quantities of glycogen added.

TABLE 1.—*Reproducibility of the method for glycogen when applied to strain 721 cells**

Sample No.	Gm. glycogen per 100 gm. protein	Deviation from mean	Percent deviation
A	2. 45	0. 05	2. 1
B	2. 41	0. 09	3. 6
C	2. 48	0. 02	0. 8
D	2. 66	0. 16	6. 4
E	2. 52	0. 02	0. 8
	2. 50	0. 068	2. 7

*Aliquots of the suspension were centrifuged down and the cells of each tube carried through the entire analytical procedure. These cells were from the experiment of 10/2/50 of table 2 on the strain 721 C3H mouse liver cells.

Hydrolysis on the above glycogen samples was carried out directly in the tube used for precipitation of the glycogen, the sulfuric acid was neutralized and the excess sulfate removed simultaneously by careful titration with barium hydroxide. A suitable aliquot of the supernatant was taken after centrifugation at 2,500 × g. In the event the end point was overstepped in the above titration, a precipitate appeared when the

sulfate-containing sugar reagent was added in the next step. Where there was little precipitate the estimation could still be saved by centrifugal removal of the barium sulfate and use of an aliquot of the remaining supernatant.

3) Collecting cells for glycogen estimation.—For the direct comparison of the glycogen content both liver cells and fibroblasts were used, both maintained in T-60 flasks (*7*) according to the procedure described by Evans *et al.* (*1*). The cells were harvested 3 hours after renewal of the feeding solution, as it was inferred from reports in the literature (*8–13*) that much of the glucose added with the fluid change would have been removed from the solution by this time and perhaps converted to glycogen. Sufficient of the large cultures were pooled so that there were approximately 1,000 mg. of the cells available for analytical work, following centrifugation to pack the cells lightly in the bottom of a tube.

4) Estimation of glucose, total nitrogen, protein, and dry weight.—For the glucose studies on the culture fluid, protein was removed by precipitation with zinc hydroxide (*14*) and subsequent centrifugation and filtration. Glucose was estimated in the filtrate by the same procedure as used for the glycogen hydrolysates. The standards were put through the barium hydroxide–zinc sulfate treatment in a fashion similar to the supernatant from the cell cultures. The Coleman Universal #14 spectrophotometer was used at 660 λ.[6]

Total nitrogen was estimated by the procedure of Hiller, Plazin, and Van Slyke (*16*), with absorption of the ammonia in boric acid and titration with a mixed indicator (*17*).

Dry weights were taken by heating the cellular mass at 100° C. to constant weight. Total protein was calculated as 6.25 times the total nitrogen obtained by the aforementioned modified Kjeldahl methods.[7]

5) Measurement of number of cells present.—The quantity of cells taken for the glucose utilization studies was ascertained by the procedure for the enumeration of cell nuclei (*18*) and application of the method for the preparation of replicate cultures (*19*) previously reported.

Results

The comparison of glycogen content for the two cell strains grown in massive culture under identical conditions is shown in table 2. The results are expressed as grams of glycogen per 100 grams of cell protein as it is believed that this gives a clearer picture of the relationship of cell mass to glycogen content than would the calculation on the total dry weight basis, following the saline wash. It is believed that in this way little error is introduced, because of the low glycogen content.[8] The glycogen content never becomes as great as that of the liver of a normal,

[6] Ringbom (*15*) curves were run at various wave lengths and 660 λ selected as that showing suitable sensitivity.

[7] We are indebted to Mr. E. Myles Glenn of the Endocrinology Branch for the Kjeldahl nitrogen analyses.

[8] Fenn and Haege (*20*) have shown, however, that in the intact animal the deposition of glycogen in the liver is accompanied by amounts of water, potassium, and chloride such that the concentration of each of these substances in the liver remains unchanged, while the protein becomes diluted to the extent that would be expected if the original absolute amount of these substances remained unchanged by the addition of glycogen and water.

TABLE 2.—*Glycogen content of strain 721 mouse liver epithelial cells and strain 929 (L) mouse fibroblasts*

Date	Strain	Protein percent wet weight (*6.25 N*)	Gm. glycogen per 100 gm. protein
10/ 2/50	721	18. 6	2. 50
	929	14. 7	0. 07
9/25/50	721	21. 4	2. 99
	929	16. 5	0. 16
9/18/50	721	17. 9	4. 30
	929	15. 6	0. 12
9/11/50	721	19. 6	4. 95
	929	15. 1	0. 13
9/ 6/50	721	16. 9	3. 45
8/28/50	721	16. 5	4. 36
	929	12. 5	0. 30
8/15/50	721	17. 4	0. 69
7/31/50	929	15. 4	0. 04
7/27/50	721	15. 0	3. 07
7/14/50	929	14. 9	0. 04
7/12/50	721	15. 0	1. 20

Average 721=3. 06
Average 929=0. 123
Ratio 721/929=24. 9

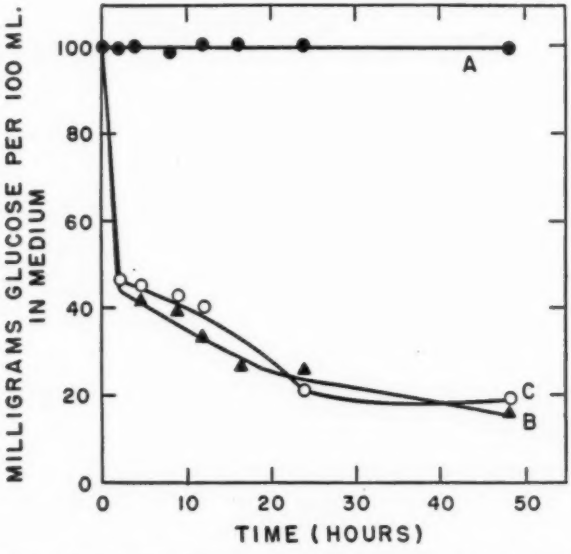
intact, fasting mouse (*cf. 21*) even in the strain 721 cells that originated from liver epithelium, but the content of the strain 721 cells is 25 times that of the strain 929 fibroblasts.

Incidental to the examination of the large cultures for glycogen content of the cells, glucose was estimated in the supernatant nutrient medium after centrifugation of the cells to remove them for glycogen analysis. Glucose had been added to the medium as prepared in order to bring the total content to approximately 100 mg. per 100 ml. of nutrient. Typical results are shown in table 3 from data obtained incidental to the experiment of 8/28/50 of table 2.

TABLE 3.—*Glucose content of used and control medium at stated intervals*

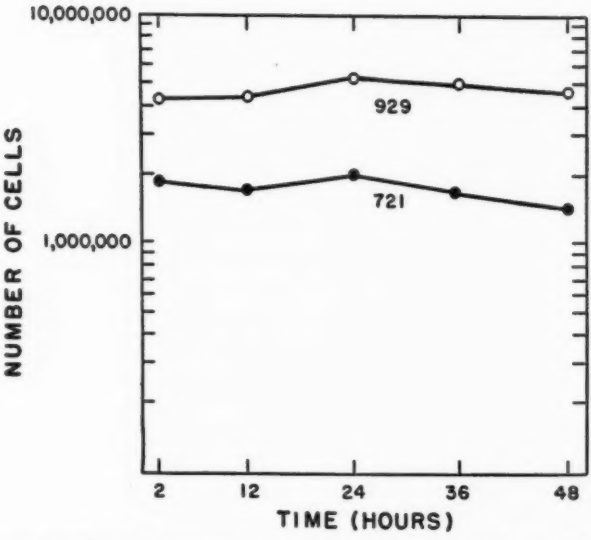
Cell strain	Elapsed time (*hours*)	Glucose content (*mg./100 ml.*)		
		"Used" medium	Control medium	
		Incubated at 37° with cells	Incubated at 37° without cells	Frozen (−5°) without cells
721	3	74	98	100
	72	0	102	100
929	3	71	100	100
	72	1	103	98

The percent glucose removed from the medium by replicate cultures of each of the two strains of cells grown in T-12 (7) flasks during a 48-hour interval succeeding renewal of the nutrient is shown in text-figure 1. Following the renewal, replicate cultures were removed and counted at 2, 12, 24, 36, and 48 hours and the results of the population count are shown in text-figure 2. The experiment was repeated with the populations adjusted to more nearly equal density and an attempt was made to follow the glycogen content of the cells under these conditions. The attempt to estimate glycogen in a single culture from one T-12 flask was never very satisfactory because the use of a single small culture drops all values for glycogen down into the region where the percentage error is large. The glucose utilization and the cell count are shown in text-figures 3 and 4, respectively. The glycogen content of the cells during this time is shown in table 4. Content is reported by T-12 flask rather than as a percent of protein or dry weight, inasmuch as the volume of work needed to set up and handle sufficient flasks to permit cell enumeration and estimation of glucose, total protein, and dry weights was such as to preclude all these. Wet weight determinations were made on the total cells in a few of the flasks from experimental series #1271, and since cell numbers were known from the nuclei counts on replicates, calculations could be made as to the weight of the individual cells. Such calculations have been made in table 5.[9]



TEXT-FIGURE 1.—Experiment 1271. Time-course of glucose content of the medium supporting the cells. A) Control, incubated medium without cells; B) supernatant medium from strain 721 cultures; C) supernatant medium from strain 929 cultures.

[9] In view of the difficulties and uncertainties connected with glycogen analyses on these small quantities of cells we have refrained from making further calculations on glycogen content of the individual small flasks.



TEXT-FIGURE 2.—Experiment 1271. Number of cells per T-12 flask during the time course of the experiment.

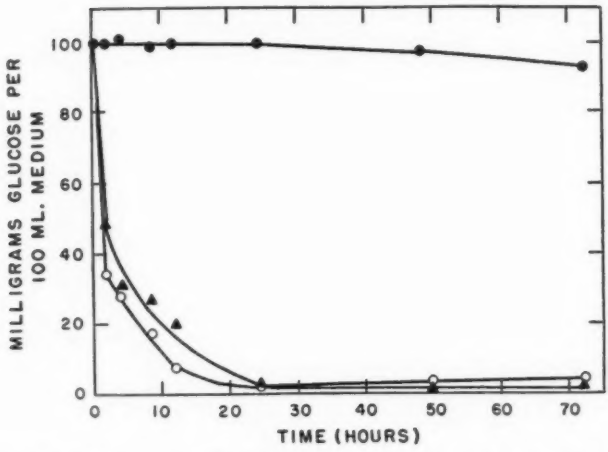
TABLE 4.—*Time-course of glycogen content of cells from individual flasks*

Time (*hours*)	Micrograms per flask	
	Strain 721*	Strain 929†
2	29	6
4	22	2
8	13	0
12	6	0
24	0	0
48	0	0
72	0	0

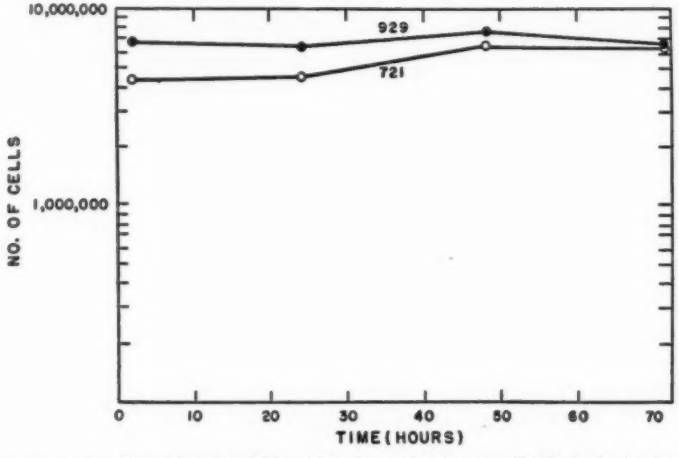
*Mouse liver epithelium.
†Mouse fibroblasts (fibrosarcoma).

TABLE 5.—*Calculation of the wet weight of a single cell of the strains 721 and 929 mouse cells*

Flask series (*No.*)	Sample time (*hours*)	Wet weight (*mg.*)	Dry weight (*mg.*)	Dry weight (*percent*)	No. of cells (*million*)	Weight of single cell $\times 10^{-9}$ (*micrograms*)
721-A-18	24	5. 5	1. 0	18. 2	2. 0	2. 75
929-B-9	24	19. 6	3. 7	18. 9	5. 3	3. 7
929-B-23	48	15. 6	3. 0	19. 2	4. 6	3. 4



TEXT-FIGURE 3.—Experiment 1322. Glucose remaining in the culture medium during a 72-hour period. ●—Control, incubated culture medium without cells; ▲—supernatant medium from strain 721 cells; ○—supernatant medium from strain 929 cells.



TEXT-FIGURE 4.—Experiment 1322. Number of cells per T-12 flask during time-course of the experiment.

Discussion

Under the conditions of the present experiments the strain 721 and strain 929 cells, maintained for years by the cell-suspension method of subculture [10] onto glass or cellophane substrate, show a glucose utilization very similar to that reported in the literature for cells maintained by the discrete explant method (*10*). There is no obvious connection between glucose utilization and growth, nor does there seem to be any correlation

[10] At the time of initiating these experiments strain 929 had been carried for 10, strain 721 for 2 years.

between glycogen content and glucose utilization since the strain 929 cells withdrew the glucose from the medium at approximately the same rate as the 721 cells of liver origin. On the basis of the lesser number of cells in the 721 flasks in both experiments, it would appear that there may be a greater glucose utilization per individual cell by the cells of liver origin.

As stated at the outset, one aspect of this study was to see if the strain 721 cells could be more clearly defined with respect to what was believed to be their origin. The greater glycogen content compared with that of strain 929 cells is compatible with the belief that the strain 721 cells are liver epithelium. The strain 929 fibroblasts are, however, descendents of a strain once found to have undergone sarcomatous change, and at the time of use in these experiments were still capable of yielding sarcoma in a very low percentage of reinoculations into mice of the same strain. It has been reported that sarcoma-bearing mice are able to deposit less glycogen (*21*) in the liver than are normal control animals. While the possibility may seem remote, it needs to be borne in mind that the strain 929 cells, by having once produced sarcomas, may have lost the power to store glycogen. Consequently the finding that the strain 721 cells have 25 times the glycogen content of the fibroblasts must not be given the weight of finality as a criterion for distinguishing these cells, especially since this content is less than that shown by the liver of the intact, fasting mouse.

The conditions chosen for these studies, *i.e.* growing the cells in standard stock medium of horse serum, chick-embryo extract, and saline, were those known to favor good growth and multiplication of the cells over long intervals as determined by counts of the nuclei. One must infer from the literature, particularly the work of Hastings' group (*22–24*), that the conditions are suboptimal with respect to the synthesis or maintenance of any considerable glycogen stores in the cells.

For the primary purpose of this study it seemed inadvisable to alter the ionic environment as drastically as appears to be called for to achieve optimal conditions for this one function. Rather it was believed that if work was to be done on the effect of the ionic environment on glycogen storage or utilization that it should constitute a separate study.

Summary

A study of the glycogen content and glucose utilization of strain 721 mouse liver epithelial cells and strain 929 mouse fibroblast cells has been made with the cells grown as suspensions under similar conditions.

There was no pronounced difference in the rate of removal of glucose from the medium by the two strains.

The glycogen content of the strain 721 cells was found to be 25 times that of the strain 929 cells. Insofar as this finding yields information as to origin it is compatible with the view that the strain 721 cells, originally taken as biopsy of liver epithelium, are still liver epithelium despite morphologic appearance.

References

(*1*) Evans, V. J., Earle, W. R., Wilson, E. P., Waltz, H. K., and Mackey, C. J.: The growth *in vitro* of massive cultures of liver cells. J. Nat. Cancer Inst. 12: 1245–1265, 1952.

(*2*) Sanford, K. K., Earle, W. R., and Likely, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246, 1948.

(*3*) Good, C. A., Kramer, H., and Somogyi, M.: The determination of glycogen. J. Biol. Chem. 100: 485–491, 1933.

(*4*) Nelson, N.: A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375–380, 1944.

(*5*) Leibson, L. G.: Glycogen content of the liver in chick embryos in various stages of incubation. Fiziol. Zhur. 36: 191–202, 1950.

(*6*) Lee, W. H.: The glycogen content of various tissues of the chick embryo. Anat. Rec. 110: 465–474, 1951.

(*7*) Earle, W. R., and Highhouse, F.: Culture flasks for use with plane surface substrate tisssue cultures. In preparation.

(*8*) Krontovsky, A. A.: Zur Charakteristik des Stoffwechsels in einzelnen Teilen des Gehirns. Versuche mit Explantaten aus dem Gehirn ausgewachsener Tiere. Biochem. Ztschr. 182: 1–10, 1927.

(*9*) Krontovsky, A. A., and Jazimiska-Krontowska, U.: Sugar consumption by tissue cultures of pure strains of fibroblasts. Zhurnal. Exper. Biol. Med. 9: 140–150, 1928.

(*10*) Holmes, B. E.: Metabolism of tissues growing in culture. Biochem. J. 27: 391–396, 1933.

(*11*) Gemmill, C. L., Gey, G. O., and Austrian, R.: The metabolism of tissue cultures of Walker rat sarcoma 319. Bull. Johns Hopkins Hosp. 66: 167–184, 1940.

(*12*) Wilson, H., Jackson, E. B., and Brues, A. M.: The metabolism of tissue cultures. I. Preliminary studies on chick embryo. J. Gen. Physiol. 25: 689–703, 1942.

(*13*) Willmer, E. N.: Carbohydrate metabolism of chick fibroblasts *in vitro*. J. Exper. Biol. 18: 237–256, 1942.

(*14*) Somogyi, M.: Determination of blood sugar. J. Biol. Chem. 160: 69–73, 1945.

(*15*) Ringbom, A.: Über die Genauigkeit der Colorimetrischen Analysenmethoden. Ztschr. Anal. Chem. 115: 332–343, 1939.

(*16*) Hiller, A., Plazin, J., and Van Slyke, D. D.: A study of conditions for Kjeldahl determination of nitrogen in proteins. J. Biol. Chem. 176: 1401–1420, 1948.

(*17*) Ma, T. S., and Zuazaga, G.: Micro-Kjeldahl determination of nitrogen. New indicator and improved rapid method. Anal. Chem. 14: 280–282, 1942.

(*18*) Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., Jr.: The measurement of proliferation of tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1950.

(*19*) Evans, V. J., Earle, W. R., Sanford, K. K., Shannon, J. E., Jr., and Waltz, H. K.: The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Cancer Inst. 11: 907–927, 1951.

(*20*) Fenn, W. O., and Haege, L. F.: The deposition of glycogen with water in the livers of cats. J. Biol. Chem. 136: 87–101, 1940.

(*21*) Young, N. F., Kensler, C. J., Seki, L., and Homburger, F.: Deposition of liver glycogen in normal mice and in mice bearing sarcoma 180. Proc. Soc. Exper. Biol. & Med. 66: 322–333, 1947.

(*22*) Hastings, A. B., and Buchanan, J. M.: The role of intracellular cations on liver glycogen formation *in vitro*. Proc. Nat. Acad. Sc. 28: 478–482, 1942.

(*23*) Buchanan, J. M., Hastings, A. B., and Nesbett, F. B.: The effect of the ionic environment on the synthesis of glycogen from glucose in rat liver slices. J. Biol. Chem. 180: 435–445, 1949.

(*24*) Teng, Ching-Tseng, Sinex, F. M., Deane, H. W., and Hastings, A. B.: Factors influencing glycogen synthesis by rat liver slices *in vitro*. J. Cell. & Comp. Physiol. 39: 73–93, 1952.

Proceedings: Tissue Transplantation Conference

Arden House, Harriman, New York
October Seventh and Eighth, 1952

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Tissue Transplantation Conference [1]

The tissue transplantation conference held at Arden House, the Harriman campus of Columbia University, Harriman, New York, October 7 and 8, 1952, was organized in response to the desire expressed by many investigators for an opportunity to get together and exchange up-to-the-minute information in this rapidly advancing field. The conference was held under the auspices of the Morphology and Genetics Study Section, Division of Research Grants, National Institutes of Health. An attempt was made to organize the meeting as a working conference in which those actively engaged in the clinical and experimental aspects of tissue transplantation could enter informally into free exchange of ideas in order to define the problems involved in transplantation of tissues in man, to ascertain what light was being shed on these problems by present experimental work, and to see what new approaches to the problem were indicated. Two days were devoted to the conference, with three sessions each day. The conference opened with a session on clinical problems, and the five following sessions bore more directly on experimental work.

Acknowledgment

The conference gratefully acknowledged the assistance of the American Cancer Society in providing the funds whereby Dr. P. J. Gaillard from Holland and Dr. P. B. Medawar from England could attend. Their active participation greatly contributed to the success of the conference.

[1] Received for publication August 14 and 21, 1953.

Program

OCTOBER 7

Morning Session—*Clinical Problems*
Moderator: Dr. A. E. Maumenee, Stanford University
Speaker: Dr. W. P. Longmire, Jr., University of California

Afternoon Session—*Initial Host Reaction to the Graft*
Moderator: Dr. H. B. Andervont, National Cancer Institute
Speaker: Dr. George O. Gey, Johns Hopkins University

Evening Session—*Genetics of Tissue Transplantation*
Moderator: Dr. C. C. Little, Roscoe B. Jackson Memorial Laboratory
Speaker: Dr. G. D. Snell, Roscoe B. Jackson Memorial Laboratory

OCTOBER 8

Morning Session—*Acquired Immunity to the Graft*
Moderator: Dr. J. Kidd, Cornell University Medical College
Speaker: Dr. E. J. Eichwald, Salt Lake General Hospital

Afternoon Session—*Methods of Conditioning the Graft*
Moderator: Dr. M. K. Barrett, National Cancer Institute
Speaker: Dr. T. S. Hauschka, The Institute for Cancer Research

Evening Session—*Methods of Conditioning the Host*
Moderator: Dr. N. Kaliss, Roscoe B. Jackson Memorial Laboratory
Speaker: Dr. H. W. Toolan, Sloan-Kettering Institute

The Homologous Transplantation of Tissues: Clinical Aspects [1]

W. P. Longmire, Jr., *Major, Medical Corps, United States Air Force*

In clinical medicine the term *homograft* is widely used to denote the tissue transplanted from one individual to another of the same species. The present evidence suggests that certain tissues that have been most frequently used clinically as homografts, such as cartilage, bone, cornea, and blood vessels, serve only as supporting structures for the invading cells of the recipient, and that the cells of the grafted tissue die. Such grafts have been found to serve a very useful purpose in clinical practice and as the principles of their preparation and preservation, as well as their limitations, are better understood, their application will undoubtedly be widened. The problems involved in this type of more or less inert graft differ in many aspects from the problem involved in the successful transfer of tissues whose cells must maintain their ability to grow and reproduce.

To clarify and emphasize this difference, the term *homostatic graft* might be applied to inert tissues such as bone and cartilage when transferred from one individual to another of the same species; and the term *homovital graft* might be used in reference to grafts whose cells must continue to grow and reproduce for the graft to be effective after similar transplantation.

There are many common problems in the study and use of these two types of homografts, but the differences in clinical application are sufficiently great to justify such a classification.

The purpose of this report is 1) to review the present status of certain homostatic grafts, and 2) to discuss the limited clinical uses and problems of homovital grafts.

Homostatic Grafts

Many surgeons, including this author, have found the results of grafting with autogenous tissues such as cartilage, bone, and fascia sufficiently superior to the results obtained by the use of similar homografted material that the patient's tissues are utilized whenever possible or practical. Although there is little statistical clinical evidence to support the impres-

[1] Presented at the Tissue Transplantation Conference, Arden House, Harriman, N. Y., October 7, 1952.

sion, autogenous grafts seem less susceptible to infection, inflammatory reaction, and absorption than comparable homologous grafts. At times, obtaining autogenous tissue may increase the magnitude of an operative procedure to such an extent that the use of homologous tissue may be indicated as a practical substitute. In other instances the lack of any available autogenous tissue for transplantation, such as in cases of corneal grafts, large bone grafts, or blood-vessel grafts, may require the utilization of homologous tissue.

Blood vessels.—Although the idea of transplanting vessels from one individual to another to bridge vascular defects has been attempted and discussed at intervals since the report of Carrel (*1*) in 1908, Blakemore *et al.* (*2*, *3*) and Gross and Peirce (*4*) reawakened current interest in the technique. In 1942, Blakemore introduced the vitallium-cuff technique to expedite blood-vessel anastomoses and suggested the use of autogenous and preserved homologous vein and artery grafts to bridge critical arterial defects, particularly in isolated vascular injuries occurring during warfare.

Gross and Peirce in 1949, studied the preservation of arterial grafts and recommended that the vessels be stored in a balanced salt solution with 10 percent plasma or serum, and with penicillin.

The reaction of the host destroys all the cells within the graft in 3 weeks, according to Swann (*5*). The elastic tissue persists for at least 2 years. Within 1 month the graft is surrounded by firm fibrous tissue and new endothelial cells grow in from the ends of the vessel to line the graft completely.

It is interesting that although all the investigators in this field are apparently agreed that this type of graft serves only as a supporting framework for the invading tissues of the host, Peirce and Gross (*6*) found a close correlation between the viability of the cells of the graft when grown in tissue culture and the successful function of the graft following transplantation. Grafts placed in balanced salt solution with 10 percent plasma and maintained at 4° C. "took" well when transplanted, and the vitality of the cells of the grafts in tissue culture was high, if they had not been stored longer than 45 days. The percentage of "takes" and the percentage of viable cells in tissue culture decreased progressively with longer periods.

Coleman, Deterling, and Parshley (*7*), who have studied the use of frozen vessel grafts, state that "viability of the aortic homograft *per se* is not a requisite for success in vascular grafts. The significant difference between the percentage of demonstrable viability and subsequent functional success leaves no alternative conclusion but that viability is not the important factor it was once thought to be. From our experience with the experimental transplantation of aortic homografts, it appears that in time the degree and type of histological changes developing after implantation will prove to be comparable regardless of what method of preservation is used. As yet, we are not prepared to predict what the ultimate degenerative effects on function will be in such trans-

plants. At present, we feel secure in stating that the frozen graft seems to hold promise for use in smaller hospitals and communities or whenever long periods of storage are anticipated before use." The above authors do conclude, however, that the graft stored in a nutrient medium is probably the most functional type at present, especially if used within the first 6 weeks of storage and if anticoagulant drugs are not to be employed. Peirce (*8*) explains this apparent superiority of the nutrient-stored graft as follows: "It is apparent that the failures of arterial homografts of all types occur early, during that time when viably implanted tissue differs in two important ways from nonviable: 1) The lining is more physiologic and would be expected to resist thrombosis. 2) Many of the cells are living and capable of participating in healing, presumably at the suture line and along the extent of the adventitia as well as in the intima. This apparently endows the grafts with considerable resistance to infection. During this (early) period a nonviable graft appears peculiarly subject to infection and attendant disruption. Possibly also, intimal changes predispose to thrombosis. A suitably preserved viable graft, on the other hand, behaves for a time much as an autograft and can actually contribute to the healing during this critical period. In addition, it maintains a viable and relatively physiologic lining. Consequently, disruption is very infrequent and thrombosis unusual."

McCune *et al.* (*9*) found that practically all of the circulation that is established in the wall of the grafted vessel reaches the graft from the surrounding tissue. There is limited vascularization from the ends of the vessel into the graft, but none from the lumen. They stress the importance of placing the grafted segment in a good vascular bed.

Certain factors considered important in utilizing vascular homografts have been enumerated by Swann (*5*). 1) The vessel to be used as a homograft should be removed from a person between 2 and 35 years of age. 2) The donor must not have died from any disease whose transmission to the recipient is a possibility. 3) The vessel should be removed within 6 hours of death. 4) Grafts preserved in a nutrient medium should not be used after they have been stored longer than 45 days. 5) A suture anastomosis should be used in inserting the graft. 6) The diameter of the graft should closely match the diameter of the recipient vessel. 7) the maximal length of vessel that may be grafted is unknown. (Swann reports a successful graft 5 inches in length.)

The appearance of calcification in the intima in some of the grafts and the development of dense fibrous tissue in the adventitia suggest that further long-term observations, over a period of several years, are necessary before forming a final evaluation of vascular homografts regardless of the method of preservation.

Cornea.—In 1948, Owens *et al.* (*10*) reviewed the results of corneal transplant operations from seven ophthalmological clinics. Of 417 corneal transplants studied, 36.5 percent remained clear. The percentage of clear grafts varied greatly with the cause of the corneal opacity. The best results were obtained in cases of keratoconus (65.2 percent), hereditary

dystrophy (58.8 percent), interstitial keratitis (49.0 percent), and nonspecific inflammatory scars (46.9 percent). Poor results were obtained in cases with active keratitis or corneal ulcer (23.1 percent), scars from chemical burns (20.8 percent), trauma (18.2 percent), or gonococcal ulcers (8.7 percent).

The larger the size of the corneal opacity and the greater the extent of corneal vascularization, the less were the chances of obtaining a clear graft. In the group of cases with the best preoperative prognosis, about two-thirds of the grafts remained clear. In the most unfavorable cases there was only one change in eight of the grafts remaining clear.

The youngest patient in this series of cases was three years of age and the oldest eighty years. However, no correlation was found between the percentage of grafts remaining clear and the age of the patient.

No statement is made in this report in regard to preparation and storage of the donor cornea. It is assumed that this factor remains relatively constant. McLean (*11*) states, "Postmortem eyes should be obtained as soon as possible after death, preferably within 4 to 6 hours and stored at about 4° C. At present the best method of storage is in a tightly sealed container in a saturated atmosphere of water vapor. The allowable time of storage has not yet been well worked out. It is best to use the donor eye as soon as possible. The best index of suitability is not the elapsed time but the condition of the cornea on close inspection. In practice, it seems to make little difference whether the cornea be [fresh] or stored, as long as its condition is good at time of operation."

Maumenee and Kronblueth (*12*) studied various methods of preserving the donor corneas in transplants between rabbits. Of 15 grafts stored in formalin, only one partially clear corneal transplant was obtained after grafting. Likewise, in a series of 13 corneal transplants in which the cells of the graft were killed by freezing, no clear grafts were obtained. For 5 to 7 days after transplantation many of these grafts looked as promising as did fresh grafts. However, all became progressively more edematous after the sutures were removed, and frequently the anterior lamellae sloughed off during the first 2 weeks after operation.

All their efforts to obtain a clear corneal graft by the use of nonviable corneas in which the cells had been killed either by freezing or by formalin were completely unsuccessful. They conclude that these experiments would indicate that living donor stromal cells, at least during the first stages of the graft, are essential for its final clarity.

Bajenova (*13*) has demonstrated that corneas preserved in moist chambers at 2° C. for 10 days contain viable stromal cells that will grow in tissue culture. The oxygen consumption and the Q_{O_2} of stored bovine corneas have been studied by Duane (*14*). He found that 1) the Q_{O_2} of the cornea remained normal when stored over Ringer's solution (at 4° C.) or under oil (at 15° C.) for seven days. The respiratory rate falls to one-fifth of normal in 10 to 12 days under these conditions. 2) Formalin inhibits respiration of the cornea immediately and this action is apparently irreversible. 3) The corneal Q_{O_2} is reduced to one-half of normal within

24 hours by quick freezing. Within four days the Q_{O_2} has fallen to one-fifth of normal, where it remains for up to 60 days' storage.

In regard to the ultimate fate of the corneal homograft, Maumenee and Kornblueth (*12*) draw the following conclusions. The majority of the stromal cells continue to live in the graft and do not show a massive degeneration or replacement. They are supplemented, at least to a limited extent, by the newly formed keratoblasts from the recipient's macrophages. The epithelium is replaced by a migration of epithelium from the recipient cornea. The endothelium may be repaired from the uninjured cells of the graft or by a migration of cells from the recipient cornea.

Maumenee (*15*) has obtained experimental evidence to support the theory that delayed opacification of the transplanted cornea develops as a result of an acquired immunity action. When skin from the same donor was used as an antigenic supplement, two weeks after a corneal transplantation, the corneal graft became cloudy in 28 of 30 eyes after insertion of the skin. As transfers of the donors' skin 6 to 8 weeks after corneal grafting into seven rabbits did not produce opacification of the cornea, it was suggested that the original donor cells of the graft had been replaced, by this time, by invading recipient cells and, therefore, the graft was no longer susceptible to the sensitivity reaction.

Infection, defects of the donor material, and poor apposition of donor and recipient tissues obviously may also be responsible for clouding of corneal grafts.

In summary, effective results have been obtained in 36.5 percent of a large series of cases in which transplants of cornea were used in a variety of pathologic conditions. The extent of opacification and vascularization of the recipient cornea is one of the most important factors governing the success or failure of corneal grafts in patients. Present evidence would indicate that viable grafts are superior to nonviable grafts, despite the fact that in certain cases the living cells in the graft may react to a delayed sensitivity state of the host, causing opacification of the cornea. A gradual replacement in the graft of all donor cells by invading host cells is probably complete within 6 to 8 weeks. In the successful graft, massive simultaneous cell destruction does not occur.

Bone.—Wilson (*16*) has reported a study of 214 patients who were subjected to 278 operations in which sterile refrigerated homologous bone grafts were used. Wound infection occurred in 4 cases (1.4 percent) and there was loss of the graft in 2 (0.7 percent).

In a follow-up to determine the final result with respect to healing of the grafts, 144 patients were traced who had undergone 179 orthopedic operations, for a large variety of conditions. Considered on the basis of the number of operations, the results were found to be successful in 80 percent, and unsuccessful in 11 percent. Fifteen patients (9 percent) had undergone operations too recently for a determination of the result. By eliminating four cases in which the failure was unrelated to bone healing, the rate of failure could be lowered to 8 percent.

From this study Wilson drew the following conclusions: 1) With careful

technique, homologous bone grafts may be preserved for long periods of time for surgical use. 2) Such grafts are well tolerated by human tissues and the risk of infection is no greater than with autogenous grafts. 3) The healing of such grafts takes place by a process of invasion, absorption, and replacement similar to that of autogenous bone grafts. 4) The results obtained are identical with those from the use of autogenous grafts, except that in some instances the healing appears to be a little slower. 5) The use of homologous bone grafts offers great advantages to the patient and the surgeon from the standpoint of availability, abundance, and the elimination of a secondary operation to obtain autogenous bone.

These experiences reported by Wilson are the most extensive thus far recorded. The reports of other surgeons concerning smaller series of cases express greater reservations regarding the use of homologous bone as a substitute for autogenous bone in grafting procedures. Speed (*17*), in discussing his experiences with over one hundred cases in which homologous bone was transplanted, states that although his results closely parallel those of Wilson, in difficult cases he prefers fresh autogenous grafts when they are available. Lipscomb (*18*) expresses the opinion of many orthopedic surgeons as follows: "At this stage of our knowledge it is felt by most surgeons using bone transplants that the autogenous graft is the one of choice in most instances, provided the operative risk and postoperative morbidity will not be seriously affected by the removal of such a graft at the time of definitive surgical treatment, and provided a sufficient supply of autogenous bone can be obtained. In those instances already mentioned in which the factors in question do not apply, the surgeon would be justified in using frozen homologous transplants or a combination of autogenous and homologous transplants."

In Wilson's cases homologous bone was obtained for transplantation from various operative procedures in which healthy, uninfected bone was excised. Bone from cadavers was not utilized. The bone was cleaned of all soft tissue and cartilage when fresh and cut into pieces of a size suitable for storage. A small piece of bone was taken for sterility studies. The graft was placed in a sealed glass jar and stored at a temperature between $-10°$ C. and $-20°$ C. The average period of refrigeration for all the bone grafts used was 69 days. Several specimens were used after preservation for more than one year. Length of refrigeration of the graft did not seem to affect the clinical result.

Reynolds, Oliver, and Ramsey (*19*) have reported their clincial and experimental experiences with homologous bone grafts preserved in merthiolate solution. The bone, obtained from suitable operative procedures and selected autopsies, was placed in a 1 to 1,000 aqueous merthiolate solution. The container was stored in an ordinary icebox, although refrigeration of this preparation is not necessary. The merthiolate solution was changed every two weeks and cultures taken of the graft. The bone was washed in sterile saline solution before it was used. They have observed no evidence of sensivity to the merthiolate, or excessive tissue reaction on the part of the recipient to the merthiolate-preserved bone. They believe

that the process of fixation and replacement is accomplished in a similar manner in autogenous and homologous bone grafts. The observations of Hatcher (*20*) suggest that some of the cells of an autogenous bone graft are capable of survival and of instigating and accelerating the process of fixation and replacement when host capillaries reach the graft. Reynolds *et al.* believe this may account both for the more rapid union of the autogenous graft as compared with homologous grafts, and for the greater number of successes with autogenous grafts.

The behavior of bone grafts preserved by drying from the frozen state, or "lyophilized," has been studied by Kreuz, Hyatt, Turner, and Bassett (*21*). Bone was prepared by slow freezing at 15° C. for four days. (They state that quick freezing to low temperatures, below −55° C., would have been better.) The specimens were placed in dry ice for the eight-hour period of shipment to the laboratory. The bone was enclosed in a block of ice and dried in a Stokes machine under high vacuum at −40° C. Approximately fourteen days were required to complete the procedure. The prepared bone could be stored at room temperature. Such a method of drying from the frozen state is said to offer two advantages, 1) quick freezing at very low temperatures minimizes protein denaturation, and 2) drying from the frozen state lessens harmful concentration of salts as the solid state is maintained throughout desiccation.

In the experimental animal, the freeze-dried bone grafts so treated were incorporated in the same manner as fresh autogenous bone grafts, but at a slightly slower rate. The freeze-dried bone was somewhat superior to frozen bone in regard to the rate of healing in the early phases. The advantage of storage at room temperatures, the potentially increased length of storage periods, the ease of transportation, and initial clinical success suggested to these authors that freeze-dried bone would be suitable for use in bone banks.

In summary, there is some question as to whether any of the cells in autogenous bone grafts survive. Most observations support the view that all viable elements of a bone graft (autogenous or homologous) die following transplantation and, therefore, little attention has been directed toward the study of methods of preserving homologous bone that would maintain viability of the cellular element. Hatcher has recently obtained evidence of proliferation of the cells lining the haversian canals in autogenous bone 7 and 12 days after grafting, indicating that certain cells in these grafts survive under favorable circumstances. This observation, if substantiated, would aid in explaining the apparent superiority of autogenous bone over homologous bone transplants.

Preservation of homologous bone by freezing at −10° C. to −20° C. or by storage in a 1 to 1,000 aqueous merthiolate solution are the methods most widely used at the present time. Grafts prepared by boiling frequently give unsatisfactory results.

Freeze-drying or "lyophilization" is considered to produce less alteration of the protein and of the salt concentration of the graft. Bone grafts preserved by this method have been reported to heal more rapidly than

frozen grafts in experimental animals but their superiority in patients has not been adequately demonstrated.

The prolonged healing phase and the difficulties of adequate histologic studies of all bone grafts make it difficult to evaluate the refinements of homologous bone transplantation.

As bone contains such a high ratio of inert to cellular matter, viability of the graft may be relatively unimportant. However, it would seem that further investigations of viable unaltered homologous bone transplants might prove profitable.

Cartilage and fascia.—Homologous grafts of cartilage and fascia have been used in clinical surgery for many years. A survey of the recent literature indicates little change in the methods of preparation, storage, or utilization of these tissues.

The ready availability of autogenous fascia, removed from the fascia lata with a modern fascial stripper or by direct excision, and the superior clinical results attained with autogenous fascia have so reduced the demand for preserved fascia that few hospitals maintain a supply today. Other autogenous tissues such as dermal grafts and full-thickness skin grafts, and nonreactive metals such as tantalum, are at present frequently utilized in operative procedures in preference to preserved fascia.

Preserved homologous cartilage is widely used in reconstructive procedures, chiefly in the maxillofacial region. The tissue is prepared and stored by methods described under the section on bone grafts. The two techniques of storage most widely used at present are: 1) storage in a temperature of $-10°$ C. to $-20°$ C., and 2) storage in a 1 to 1,000 aqueous merthiolate solution.

The incidence of infection and absorption is somewhat higher when preserved cartilage is used instead of autogenous cartilage; however, obtaining an autogenous graft may frequently convert a minor surgical procedure that may be performed under local anesthesia into a major operation requiring a general anesthetic. In such cases homologous grafts are generally considered a practical substitute for autogenous tissue.

Nerve.—Davis and Ruge (*22*) have reported the results following the transplantation of nerve homografts in cats and in patients. They state, "Many investigators have tried and discarded this procedure, maintaining that the graft produces a foreign-body reaction in the host and is reduced to a fibrous band or is absorbed entirely."

Experiments were performed on 35 cats, using 2.5 cm. of fresh sciatic nerve homografts. Union of the graft to the nerve end was accomplished with the plasma clot and silk suture techniques. Microscopic examination of sections of the graft indicated that the superficial layers of the graft retained their viability, whereas the deeper areas were often necrotic. Where grafts were viable, the original fasciculi were unchanged and the endoneural tubes remained. Regeneration of the grafts was always spotty but adequate in the peripheral layers.

One year following insertion of the graft all animals were capable of nearly all motions of the involved foot and toes, with the occasional

exception of dorsiflexion of the toes, which was the last movement to return. At this time all animals responded to compression of the involved toes by prompt withdrawal of the entire lower extremity.

Homologous nerve grafts were performed on 10 patients who had been followed from 6 months to 16 years. The grafts were taken from the sciatic nerves of recently amputated extremities or from cadavers soon after death, and refrigerated for 30 minutes to 45 hours before being used. In regard to the results they state, "While none of the patients have shown any return of motor function or enough evidence of regeneration to approach normal, some have shown minimal evidence of recovery. For 2 patients it is too early to evaluate the results. Of the remaining 8 patients, 6 have shown minimal evidence of recovery and 2 have shown no sign of regeneration." Three of the nerve grafts were exposed and examined 4, 4½, and 8 months following transplantation and in no instance had the graft been absorbed or reduced to a fibrous band.

In attempting to explain the markedly inferior results in man, compared with the experimental results, the authors emphasize the importance of the condition of the graft itself. In the experimental study the grafts were removed and transplanted immediately following the death of the donor, and the diameter and length of the grafts were smaller than those used in the human beings. They indicate that attempts should be made to simulate the experimental conditions as closely as possible when employing nerve homografts clinically.

Trachea.—The transplantation of tracheal segments should also probably be included in the *homostatic* group of grafts. As the cartilaginous rings form such a large and important section of the wall of the trachea, it has been suggested that segments of the trachea might be transplanted in much the same manner as cartilage. If the cartilaginous framework of the tracheal wall could be preserved, the rapid regeneration of the tracheal epithelium, as demonstrated by Daniel (*23*), might be expected to form a new lining for such a homostatic transplant. In 1948, complete sections of the trachea including three cartilaginous rings were homotransplanted in dogs (*24*). Such grafts seemed to function well for a period of 10 to 16 days, but in all cases softening of the graft occurred with progressive collapse and stenosis. Connolly and Richards (*25*) have subsequently published their findings in a series of 8 tracheal transplants in dogs. One animal was sacrificed 8 months after transplantation. Although the dog had been well, the graft was markedly constricted and contracted and the cartilage degenerated. A second animal sacrificed 1 year after transplantation had a normal, well healed graft as seen by both gross and microscopic examination. The grafts were unsuccessful in the other 6 animals. They concluded that, 1) the usual course of a tracheal graft is degeneration of the cartilage with replacement by fibrous tissue, and 2) under "certain conditions" a homologous tracheal graft will survive in a recipient dog.

The uncertain results in experimental animals have discouraged any attempts to utilize tracheal homografts in patients.

Summary.—A review of the development of the use of various homo-

static grafts reveals that many common problems have been encountered in the transplantation of such dissimilar tissues as cornea, blood vessels, and bone.

With all types of tissues, autogenous grafts remain the most satisfactory as far as the "take" of the graft and its successful function are concerned. Current investigations would seem to indicate that the transplanted cells in "vital" homostatic grafts may actually participate in the early phases of healing, and that methods of preparation and preservation that protect the vitality of the graft should be investigated further.

The importance of the vitality of the homostatic graft to its ultimate clinical success may vary from one tissue to another, depending in part upon the ratio of cellular to inert substances of the graft and also upon how closely the graft must retain its original structure to function adequately. A corneal transplant can undergo little alteration from its original state and be successful. On the other hand considerable change may occur in and about the bone or cartilage transplant without destroying a final successful clinical result.

Methods of storage that not only render the graft nonvital but also grossly distort its chemical or physical composition, such as boiling or formalizing, have proved unsatisfactory and have been universally discarded.

Other methods of storage such as freezing, lyophilizing, or storing in merthiolate solution have the advantage of being more practical methods of storing quantities of tissues over long periods of time. Any method that preserves the "vitality" of the graft must produce sufficiently superior results to outweigh the practical advantages of other methods.

Homovital Grafts

The successful transplantation of homovital grafts remains a challenging unsolved problem. Preliminary reports of prolonged survival of skin homografts following treatment of the recipient with ACTH have not been substantiated by further studies (*26–29*). There have been reports of minor delays in the disintegration of homografts in the rabbit, but this may in part be an interference with healing rather than delayed destruction. It is interesting in this regard that in skin homografts between 1-day-old chickens we (*30*) could detect no increase in the percentage of permanently surviving grafts when the recipient was treated with ACTH. However, with injections of cortisone the surviving grafts were increased from 9 percent for the control group to 20 percent. As stated, however, no beneficial effect from the injection of either cortisone or ACTH on homografts in human beings has been demonstrated.

Allen *et al.* (*31*) have prolonged the survival time of skin homografts in the rabbit from 7 to 23 days by a preliminary desensitizing procedure. The recipient was treated with a skin antigen prepared from the full-thickness skin of the proposed homograft donor. Injections of antigen were given intravenously, intraperitoneally, and subcutaneously in divided doses over a period of 3 weeks. They concluded that their findings sup-

ported the immune theory as the mechanism of homograft destruction.

The only clinical uses for skin homografts at the present time are: 1) In the rare case of an extensive burn where skin homograft "dressings" may be used during an interim period to build up the patient's general condition, or 2) as a replacement tissue in small vital areas when autografting is contraindicated.

If one considers the ultimate destruction of homografts a result of failure of the small nourishing arterioles that grow into the graft, then there might be reason to believe that an entire organ such as a kidney or a lung, with large vascular channels connecting it to the body, would survive homotransplantation when a skin graft would not.

Carrel (*32*) in 1907 transplanted both kidneys with attached renal vessels and ureters in cats. Of nine animals that survived the operative procedure, two lived in apparent good health for approximately 1 month. Both these animals apparently died of an acute interstitial nephritis. Many plasma cells were noted in the interstitial tissues of the kidney in one of them.

Lawler *et al.* (*33*) have reported the homotransplantation of a kidney in the human with evidence that the transplant remained functional. However, at re-exploration 5 months after the original operation, the homograft was diminished in size. The ureteral and pelvic structures were absent. The blood supply was present. It was felt that the organ was alive but was not producing urine. A similar case has been reported by Servelle *et al.* (*34*). In this case the ureter was connected to the skin. Urine excretion increased from 20 cc. on the 1st day to 192 cc. on the 16th day. On the 19th day the urinary output of the grafted kidney was 600 cc. On that date the patient died of shock.

Experimental transplantation of the lung has also been reported.

Further experimental studies of such organ transplants, with careful histologic study of the graft after its ultimate fate has been established, would seem indicated. However, there seems to be no reason to recommend or to attempt such organ transfers in patients at the present time.

Summary

Classification of homografts as either homostatic or homovital grafts is suggested. Continued growth and reproduction of the cells of the graft after transplantation constitute the requirements for a homovital graft. The homostatic graft fulfills its purpose by serving as a nonreactive scaffolding for the invasion of the cells of the host.

The present status of certain homostatic grafts has been discussed, and certain current investigations concerning homovital grafts reviewed.

References

(*1*) Carrel, A.: Result of transplantation of blood vessels. J. A. M. A. 51: 1662–1667, 1908.

(*2*) Blakemore, A. H., Lord, J. W., Jr., and Stefko, P. L.: The severed primary artery in the war wounded: A non-suture method for bridging arterial defect. Surgery 12: 488–508, 1942.

(3) BLAKEMORE, A. H., LORD, J. W., and STEFKO, P. L.: Restoration of blood flow in damaged arteries; further studies on a nonsuture method of blood vessel anastomosis. Ann. Surg. 117: 481–497, 1943.

(4) GROSS, R. E., BILL, A. H., JR., and PEIRCE, E. C., II: Methods for preservation and transplantation of arterial grafts: Observations on arterial grafts in dogs; Report of transplantation of preserved arterial grafts in 9 human cases. Surg., Gynec. & Obst. 88: 689–701, 1949.

(5) SWANN, H.: Arterial grafts. Surg., Gynec. & Obst. 94: 115–117, 1952.

(6) PEIRCE, E. C., II, GROSS, R. E., BILL, A. H., JR., and MERRELL, K., JR.: Tissue-culture evaluation of viability of blood vessels stored by refrigeration. Ann. Surg. 129: 333–348, 1949.

(7) COLEMAN, C. C., JR., DETERLING, R. A., JR., and PARSHLEY, M. S.: Experimental studies of preserved aortic homografts. Ann. Surg. 134: 868–877, 1951.

(8) PEIRCE, E. C., II: The use of viably preserved tissue for homologous arterial grafts. Ann. Surg. 136: 228–238, 1952.

(9) MCCUNE, W. S., THISTLETHWAITE, J. R., KESHISHIAN, J. M., and BLADES, B.: The nutrition of blood vessel grafts. Surg., Gynec. & Obst. 94: 311–316, 1952.

(10) OWENS, W. C., FRANK, J. J., LEAHEY, B., MESSIER, P. E., SCHEIE, H. G., VINCENT, B. R., WADSWORTH, J. A. C., and STANSBURY, F. C.: Symposium; corneal transplantation; results. Am. J. Opth. 31: 1394–1399, 1948.

(11) MCLEAN, J. M.: Symposium; corneal transplantation; technique. Am. J. Opth. 31: 1370–1374, 1948.

(12) MAUMENEE, A. E., and KORNBLUETH, W.: Symposium; corneal transplantation; physiopathology. Am. J. Opth. 31: 1384–1393, 1948.

(13) BAJENOVA, M. A.: Culture des tissues de la cornée conservée. Arch. d'Opht. 53: 300–307, 1936. [Cited by Maumenee and Kornblueth (*12*).]

(14) DUANE, T. D.: The respiration of the stored cornea. Am. J. Opth. 31: 1400–1404, 1948.

(15) MAUMENEE, A. E.: The influence of donor-recipient sensitization on corneal grafts. Am. J. Opth. 34: 142–152, 1951.

(16) WILSON, P. D.: Follow-up study of the use of refrigerated homogenous bone transplants in orthopedic operations. J. Bone & Joint Surg. 33-A: 307–322, 1951.

(17) SPEED, J. S.: Discussion of the paper by WILSON, P. D.: Follow-up study of the use of refrigerated homogenous bone transplants in orthopedic operations. J. Bone & Joint Surg. 33-A: 322–323, 1951.

(18) LIPSCOMB, P. R.: The bone bank. Surg., Gynec. & Obst. 89: 485–487, 1949.

(19) REYNOLDS, F. C., OLIVER, D. R., and RAMSEY, R.: Clinical evaluation of the merthiolate bone bank and homogenous bone grafts. J. Bone & Joint Surg. 33-A: 873–883, 1951.

(20) HATCHER, HOWARD: Personal communication, quoted by REYNOLDS, F. C., OLIVER, D. R., and RAMSEY, R.: *Ibid.*

(21) KREUZ, F. P., HYATT, G. W., TURNER, T. C., and BASSETT, A. L.: The preservation and clinical use of freeze-dried bone. J. Bone & Joint Surg. 33-A: 863–872, 1951.

(22) DAVIS, L., and RUGE, D.: Functional recovery following the use of homogenous nerve grafts. Surgery 27: 102–114, 1950.

(23) DANIEL, R. A., JR.: Regeneration of defects of trachea and bronchi. J. Thoracic Surg. 17: 335–349, 1948.

(24) LONGMIRE, W. P., JR.: Repair of large defects of the trachea. Ann. Otol., Rhin. & Laryng. 57: 875–883, 1948.

(25) CONNOLLY, J. E., and RICHARDS, V.: The homotransplantation of the trachea in dogs. *In* Surgical Forum—Clin. Cong. Am. Coll. of Surg. Philadelphia, W. B. Saunders Co., 1950, pp. 47–53.

(26) MARRANGONI, A. G.: The effect of cortisone on skin transplants in the experimental animal. *Ibid.*, 1951, pp. 462–468.

(*27*) Randall, P., Brown, J. E., McDowell, F.: The effects of ACTH and cortisone on experimental skin homografts. *Ibid.*, 1951, pp. 475–481.

(*28*) Trusler, H. M., Glanz, S., Bauer, T. B.: The use of ACTH in the treatment of extensive burns: Relationship to homologous skin grafting. *Ibid.*, 1951, pp. 481–488.

(*29*) Ellison, E. H., Martin, B. C., Williams, R. D., Clatworthy, H. W., Hamwi, G., and Zollinger, R. M.: The effect of ACTH and cortisone on the survival of homologous skin grafts. Ann. Surg. 134: 495–505, 1951.

(*30*) Cannon, J. A., and Longmire, W. P., Jr.: Studies of successful skin homografts in the chicken. Ann. Surg. 135: 60–68, 1952.

(*31*) Allen, H. W., Williams, R. D., Lovingood, C. G., Ellison, E. H.: The effect of donor skin desensitization and ACTH on survival of skin homografts in rabbits. Ann. Surg. 135: 239–244, 1952.

(*32*) Carrel, A.: Transplantation in mass of the kidneys. J. Exper. Med. 10: 97–140, 1908.

(*33*) Lawler, R. H., West, J. W., McNulty, P. H., Clancy, E. J., and Murphy, R. P.: Homotransplantation of the kidney in the human. J.A.M.A. 147: 45–46, 1951.

(*34*) Servelle, M., Soulie, P., Rougeulle, J., Delahaye, G., and Touche, M.: Kidney transplantation. J.A.M.A. 146: 393, 1951.

Discussion: Clinical Problems

Moderator: Dr. A. E. Maumenee, Stanford University

DR. P. J. GAILLARD, UNIVERSITY OF LEIDEN, LEIDEN, HOLLAND: With respect to skin transplants, we have had experience in grafting full-thickness human embryonic skin on large-scale burn wounds in four patients. In the middle of the wound some five or six strips of skin were grafted. The grafts took initially. The fragments fused and grew so as to cover quite an area within a period of approximately one month. Then autologous Thiersch skin grafts were made on the rest of the wounds. After they had taken, the autologous skin started to attack the embryonic skin, which slowly decreased its area, as if some kind of incompatibility existed between the two types of tissue. Nevertheless, parts of the *embryonic* skin remained intact for as long as 6 to 7 months, which is much longer than adult homografts generally do.

Another problem of interest to us was that of transplanting cultured parathyroid tissue derived from newborn humans to patients suffering from postoperative tetany. Since 1941 thirty patients have been treated. The grafts were put into the perivascular lymph spaces around the axillary arteries or veins. The patients were carefully screened first, to be sure a parathyroid deficiency really existed. Blood calcium and phosphorus estimations were made at intervals of some weeks. So far seven complete cures have been obtained, and one has been observed for 11 years. As far as the age of the hosts is concerned, it became clear that important differences existed. In the 16- to 26-year age group, five of ten cases were positive. In the 26- to 36-year age group there were two positive and two partial results from eight cases. All twelve patients in the 36- to 56-year age group were negative. In all cases we waited for at least one year before judging the effects.

Summarizing, I think these results show that transplantation of real value can be done with homografts of cultivated parathyroid tissue derived from newborn human beings.

Dr. Jonathan E. Rhoads, University Hospital, Philadelphia, Pa.

There still seems to be reasonable doubt as to whether methods can be developed in the near future to permit homografts of human skin to take. Therefore, we have been interested in methods for making more efficient use of skin available for autografts. Dr. Harold Zintel developed a method for resplitting split-thickness grafts taken with a Paget dermatome. The method consisted of cutting a rather thick graft of about 16/1000 of an inch and then, while the skin was still glued to the drum, resetting the instrument at half this thickness and recutting it. Such grafts can be used either in sheets or as postage-stamp grafts. Postage-stamp grafts from the deeper layer could be successfully grafted either right side up or upside down. Dr. Henry Royster has practiced the use of homografts interspersed with autografts to cover extensive denuded areas when sufficient skin for autografting was not available. In this situation, as the homografts disintegrated, the autografts spread out to take their place.

The variability in the duration of homografts in clinical work should be stressed, as it is not unusual for such grafts marked with an identifying tattoo to remain in place for three months or even longer. Dr. Zollinger's group, in the Department of Surgery at the Ohio State University, reported a patient who received homografts at 2-week intervals; the first of these grafts separated in about 11 weeks and the remainder of the grafts all separated within a short time thereafter, suggesting that a change had occurred in the host.

Dr. Blair O. Rogers, Presbyterian Hospital,[1] New York, N. Y.

For the past year we have been homotransplanting experimentally with skin, using human volunteers rather than animals for our research. We have transferred uniformly sized, full-thickness skin homografts from the anterior thigh region of the donor patient to newly created, asceptic, surgical defects of the anterior thigh of the host patient. All remnants of subcutaneous fat were trimmed from the under surface of the dermis of each homograft. Autografts of the same size and grafted into the same locations were permanently successful. These autografts therefore served as our controls, making us feel certain that at least the technical procedure in working with full-thickness skin homografts was a sound one.

Biopsies of these skin homografts were taken with a Reese punch biopsy apparatus. We felt that this biopsy technique did not disturb the graft itself (as it did not create any motion of the graft on the bed to which it had been applied), since the graft is firmly anchored in its "initial" take to the host bed by the time the first biopsy specimen is taken.

We have attempted within the past year to investigate, under control conditions, the basic patterns for the behavior of skin homografting in humans, and thereby have tried to verify or refute Professor Medawar's work in rabbits. In this set of experiments, in which we used grafts of standard size that were equal in width (the same diameters) and were of uniform thickness, the mean survival time of these grafts in ten cross homograftings was fairly constant and almost identical in each case. These results in humans, therefore, seem to agree with Professor Medawar's "dosage" phenomenon in rabbits.

We feel that ACTH has been amply demonstrated in both humans and animals to have no effect whatsoever in increasing survival time of skin homografts. It is possible that cortisone, because of its favorable effect in animals, might have the same favorable effect of increasing the survival time of homografts in humans if it is tried *under control conditions* such as those we have used in our human experiments.

Because the initial case of Whitelaw (2-degree and 3-degree burns treated with ACTH) died a year later under peculiar circumstances, though in apparently good health prior to his demise, and because autopsy showed plugging of his kidneys with a proteinlike sludge (this report came to us from a member of the National Research Council), we would strongly advise caution in the future use of ACTH on any skin-grafted patient, whether autografted or homografted. Certainly the side effects of ACTH on diseases such as peptic ulcer, tuberculosis, and hypertension are contraindications enough for its use in any patient in whom previous medical histories are even slightly suggestive of such diseases.

Dr. Wilford B. Neptune, Hahnemann Medical School, Philadelphia, Pa.

I agree with Dr. Longmire that clinical application of lung transplants is certainly a long way off. However, from a technical standpoint I feel we have demonstrated that it is feasible, since at the present time we have an animal that has been observed for more than one year following autogenous reimplantation of the left lung, and all studies, including X ray and bronchospirometry, are normal. Of the homologous grafts from one dog to another, our average survival time has been nine days; without the use of ACTH it was six days and with the use of ACTH it increased to 25 days.

Although we are at a loss to explain the results, we have two animals that survived for 27 days and 42 days, respectively, with histologic studies on the lung tissue giving relatively normal results. One animal died of distemper and the lung of the other animal was removed following atelectasis due to bronchostenosis. We feel sure that had these animals survived longer, the ultimate outcome would have been the same. However, we would like someone to explain these two results.

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Dr. David M. Hume, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.[1]

We have made the following observations in six human kidney homotransplants:

1) Three of the six failed to show evidence of function. In two of these the grafts were taken from donors whose blood type differed from that of the recipient's. One was a technical failure.

2) Three of the transplants showed measurable function for a limited period. In these patients the blood types of the donor and recipient were compatible as to ABO and Rh factors.

3) If the renal homografts could continue to function in the host at the level they are able to achieve temporarily, they would maintain the host indefinitely.

4) The immune reaction to kidney homografts in the human is different from that in the dog, and function in the human persists longer than that in the dog. In the human the graft goes through a period of anuria immediately following transplantation which lasts 9 to 14 days. Maximal function is then attained between 18 and 33 days, with function declining after this time. Histologically, the grafts appear quite normal for one month, after which tubular necrosis and infection gradually destroy them. Interstitial round-cell infiltration, arteritis, and glomerular damage (seen after homotransplantation in the dog) are not prominent morphologic features in human renal homotransplants.

5) Survival and temporary function of a human renal homograft deprived of blood supply for three hours and twenty minutes was noted in one case.

6) The question is raised as to whether the anoxia and acute tubular damage in some way decrease the rapidity and severity of the immune response to human renal homografts.

Dr. James W. Pate, Naval Medical Research Institute, Bethesda, Md.

It has been our experience, based on some 140 aortic grafts, 210 bone grafts, and other laboratory studies that the problems of vascularization and viability are overemphasized. In one group of our experiments we found that of 25 fresh aortic homografts, which were viable at the time of implantation, only 79 percent gave excellent clinical results; whereas of 65 freeze-dried grafts, which were completely dead at the time of implantation, 98 percent gave clinically excellent results. The latter vessels were not revascularized. This shows that there is no necessity for viability in order for a graft of the homostatic type to yield good clinical results. The microscopic findings confirm this and, in fact, show better results in the dead freeze-dried grafts.

Electric-potential studies by Dr. Sawyer have shown that injured aortic tissue and all fresh aortic grafts give off injury potentials that predispose to thrombosis. This is not the case in the freeze-dried tissues, which, being dead, are incapable of injury potentials.

These studies have left us with the impression that viability is not only unnecessary in aortic grafts, but is actually undesirable.

Dr. Herbert Conway, The New York Hospital, New York, N. Y.

The problem of permanently successful homoplasty of skin grafts is one of utmost importance in clinical practice. Clinicians regularly meet patients in whom severe burns have damaged such a high percentage of the surface area of the body that there are few or no available donor areas. The use of homoplastic skin grafts in such cases as well as in patients who have reached a state of critical illness from persistent large, open wounds (to the state of chronicity) is practiced regularly, but it is regretted that such skin grafts survive in the human for only 5 or 6 weeks. After such lapse of time it is necessary to proceed with the coverage of the wound by the use of autogenous grafts. In civilian life this situation is very real and is met in everyday hospital practice.

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In military experience the need is even greater and, in the event of disaster from atomic bombs, the need would be multiplied many thousands of times.

To be able to procure from a skin bank homoplastic skin grafts that would be permanently successful represents an ideal in surgical achievement, the value of which is immediately apparent. Diminished operative time and lessened operative shock to the patient would result.

In addition, there is a group of patients in whom generalized dermatitis, such as ichthyosis, precludes the use of the patient's own skin for wound coverage. Such patients not infrequently develop carcinoma on the surface of the body which requires wide excision and subsequent coverage by a graft. Only permanently successful homografts of skin can answer this problem. In patients who are in acute blood crises in association with diseases of the blood such as hemophilia, there is a very real field of usefulness for homografts of the skin. A hemophiliac with an open traumatic wound more often than not dies as a result of it. The hesitancy of clinicians to cause further bleeding by cutting an autogenous skin graft is easily understood. In such cases permanent skin homoplasty would offer the ideal method of management.

Conditions of lesser importance include those in which a chronic disease of the skin has affected all of the facial area and in which, for the sake of appearance, it would be desirable to transplant, permanently and successfully, skin of a similar texture and quality, *i.e.*, facial skin, for permanent coverage. Such transplants, of course, would have to come from stillborn babies or be taken from cadavers soon after death. Since refrigerated autografts of skin may be transplanted successfully as late as thirty days after cutting, it is likely that such homotransplants would survive the period of time necessary for their procurement and placement. Ultrarapid freezing and vacuum packing of skin grafts broadens considerably the probable field of usefulness of stored grafts.

The problem is to establish skin homoplasty with permanent survival of the grafts. Epithelialization is not enough, for the functions of the skin are not limited to those of cellular coverage. Elasticity, thermo-regulatory activity, pigmentation, and diaphoresis are only a few of the cutaneous functions for the preservation of which permanent survival of dermis is desirable. The skin then should be looked upon, not as one of the simplest organs of the human body, but as one of the most complex and one of the largest organs of the body.

In our animal experiments, the fact that multiple minute thromboses of the recipient bed precede the dissolution of homoplastic skin grafts led to our observations that the same process develops in the human. Further animal experimentation has shown that these thromboses are prevented and that homoplastic skin grafts become stromatized if the skin is prepared for homotransplant by primary subcutaneous burial in the donor. Such preparation results in limitation of the keratinization and atrophy of the adnexa of the skin. This approach to the solution of permanently successful homotransplants of skin will be carried over into the field of clinical investigation in man.

During this first session, variation in definition of certain terms became apparent. An informal committee consisting of Dr. Herbert Conway, Dr. H. S. N. Greene, and Dr. P. B. Medawar brought forth the following definitions, presented at a subsequent session:

Take: stromatization of the graft.

Survival of a homograft is the endurance of the graft in a state comparable to that of an autograft.

Degeneration of a homograft begins at the onset of inflammation or destructive changes that may or may not progress to completion.

After considerable discussion, however, it was agreed by the conference that each speaker or discussor should define his own terms when presenting his paper or his comments.

Discussion: Initial Host Reaction to the Graft*

Moderator: Dr. H. B. Andervont, National Cancer Institute

DR. GLENN H. ALGIRE, NATIONAL CANCER INSTITUTE, BETHESDA, MD.: I should like to present certain observations from our transparent-chamber studies bearing on the problem of the initial host reaction to the graft.

First, regarding the question of "takes": from the experimental viewpoint, I would suggest that there is only one basic question at issue here. How long do the cells of the graft survive? Survival cannot be determined solely or with precision by gross appearance, by union of graft with host tissue, by stromatization, or by vascularization of the graft. Even histologic examination of fixed material is not always sufficient. One must know the duration of survival of various cell types within the graft, as determined by growth or migration of cells on return to the original host in the case of homografts, and by cell mobility or evidence of function or cellular outgrowth when the graft is returned to tissue culture.

A concept that has persisted through the past fifty years of cancer research is that the stroma and blood vessels of the tumor graft completely degenerate and are entirely replaced by a new blood supply from the host. It was thought by Bashford, Murray, and Cramer,[1] on the basis of careful histologic studies, that transplanted tumors provoke a "specific stromal reaction" on the part of the host that results in the rapid penetration of the tumor graft by fibroblasts and blood vessels derived from the host. Others, including Algire and Chalkley,[2] have postulated a hypothetical substance produced by the tumor that stimulates rapid capillary proliferation from the host.

These interpretations are not correct. They are incorrect because the methods of static histology are inadequate to resolve such dynamic questions. On the contrary, the vascular reaction of the host in transplantation is little different whether the graft is living or dead tissue, normal, embryonic, or neoplastic tissue, or whether the stimulus to the host tissue is a foreign body, a wound, or a burn. In all cases, capillary outgrowth from host vessels is not observed in less than 5 or 6 days. I do not know of any stimulus or agent that will hasten this process. Several procedures, however, may delay it. On the other hand, it has been recognized by many workers that transplants of tumors or of embryonic organs are frequently vascularized in 1, 2, or 3 days. How then do we reconcile this fact with the preceding statements? This leads us to a second point. The graft, if derived from a growing tissue, is not passive, but usually plays an active role in its own initial vascularization. The blood vessels of such growing tissues as tumors or embryonic organs do not completely disintegrate, nor are they completely replaced by new vessels from the host. Very frequently, some of them are used again, and under favorable conditions a small implant of an embryonic organ may completely re-establish its original blood supply through the original channels in less than 24 hours. This is accomplished by the outgrowth of endothelial sprouts from blood vessels of the graft. These capillary outgrowths migrate rapidly to make connections with the vessels of the host. This is frequently the "initial vascularization" of the graft, occurring within the first few days after transplantation. It is clearly separated in time from capillary growth from the host, and it does not always occur. Connective tissue barriers may intervene, vascular remnants may not be present in the

*Dr. George O. Gey did not submit a manuscript or abstract of his address, "Initial Host Reaction to the Graft."

[1] BASHFORD, E. F., MURRAY, J. A., and CRAMER, W.: The growth of cancer under natural and experimental conditions. Imp. Cancer Res. Sci. Rep. 2, part 2, 24-29, 30-39, 1905.

[2] ALGIRE, G. H., and CHALKLEY, H. W.: Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. J. Nat. Cancer Inst. 6: 73-85, 1945.

graft or suitably oriented in relation to the vessels of the host, central necrosis or fibrin deposition may block vascular connections.

Surgeons have recognized for years the importance of activation of the vascular bed upon which the graft is to be placed. As Rous [3] has pointed out in a recent article, they have rarely considered the activation of the graft. The point I wish to stress is that the graft is not a passive recipient of vessels from the host, but in the case of actively growing tissues plays an important role in the establishment of its own blood supply.

Dr. Herbert Conway, The New York Hospital, New York, N. Y.

I should like to make some comments on the development of circulation in autogenous and homologous grafts. In microscopic observations on the development of circulation of autografts and homografts of skin in experimental animals, the tissue-chamber technique as developed by Algire and Legallais and modified by Joslin has been employed. Observations have been made during the critical 15-day period after transplantation, the period during which the success or failure of the graft is determined. The grafts survive by plasmatic circulation during the first week. Capillary budding develops at the margins and at the base of the graft. The parallelization of vessels develops an areola about the graft.[1] This control pattern was not exhibited when homologous grafts of skin were transplanted. The parallelization of new vessels was absent, there was turbidity of the plasmatic exudate, multiple thromboses developed in the tissues of the recipient bed, the capillaries failed to invade the homoplastic graft, and final thinning of the graft developed antecedent to its complete necrosis.[2] When mice in which homotransplants of skin were done were treated with corticotropin, thrombosis failed to develop in the majority of experiments but capillary budding was sluggish and the neocirculation failed to grow toward the graft. Despite therapy with ACTH that blocked the thromboses in the recipient bed, the homoplastic transplants all failed.[3]

The consistent thrombosis of the blood vessels of the recipient bed in mice on which homoplastic grafts of skin had been transplanted led to the use of an anticoagulant, dicumarol. Oral administration, according to the method of Overton, was used. The recipient mice were fed 2 mg. of dicumarol daily for approximately 4 days, at which time their prothrombin times were found to be doubled. A maintenance dose of 2 mg. of dicumarol every third day was found to sustain the prolonged prothrombin time. After 2 to 4 days of dicumarol therapy, homoplastic grafts of skin were transplanted to the dorsum of the treated animals. Donors were unrelated mice. The transparent-chamber technique was used. The development of collateral vessels and the rate of flow of blood to the grafts were observed to be more rapid and more complete than in autogenous grafts. Circulation in the grafts was active; and it was sustained for only 12 days after transplantation in some animals but for as long as 21 days in others. Six mice in this series showed active circulation of the blood vessels of the recipient bed for as long as 12 days following transplantation. By that time the transparent-chamber splints of three of the animals had pulled loose. In the three others, progressive opacity of the exudate indicated the onset of thrombosis. In one animal the profusion of new vessels and the active flow of circulation were startling. Many new vessels had grown about the margins of two-thirds of the graft, in circumferential laminations. However, the direction of their growth was not toward the graft (as in the pattern of circulation in autogenous grafts) but rather

[3] ROUS, P.: The activation of skin grafts. J. Exper. Med. 83: 383–400, 1946.

[1] CONWAY, H., STARK, R. B., and JOSLIN, D.: Observations on development of circulation in skin grafts; physiologic pattern of early circulation in auto-grafts. Plast. & Reconstruct. Surg. 8: 312–319, 1951.

[2] CONWAY, H., JOSLIN, D., REES, T. and STARK, R. B.: Observations on the development of circulation in skin grafts. III. Morphologic changes observed in homologous skin grafts. Plast & Reconstruct. Surg. 9: 557–564, 1952.

[3] CONWAY, H., STARK, R. B., and JOSLIN, D.: Observations on the development of circulation in skin grafts. IV. Effect of corticotropin (ACTH) on homologous skin grafts. Plast. & Reconstruct. Surg. 10: 67–71, 1952.

around it. In spite of the abundance of new vessels about this homoplastic graft of skin, the graft was not successful.

We were interested in studying what role, if any, the skin appendages play in the problem of homotransplantation of skin. We attempted to produce atrophy of the appendages by burying skin in a mouse, recovering this skin at a later date, transplanting it to a second mouse of high genetic diversity, and observing the graft under the transparent chamber.

The technique of burying grafts was simple. Pieces of whole-thickness skin were excised from the backs of black mice. The surrounding skin was then undermined and the free grafts buried subcutaneously. The surrounding skin was sutured over the grafts so that they were buried completely. These buried autogenous grafts were then recovered at intervals of 7, 10, 13, and 17 days. After recovery they were transplanted to white mice. We have found it extremely difficult to recover skin buried for longer than 3 weeks, since by that time the grafts have been altered to such a degree that they cannot be identified. Biopsy of such buried grafts showed that the effect of burial was apparent in the almost complete loss of keratin, the thinning of the epidermis, and the nearly complete atrophy of the adnexa (hair follicles, sweat glands, and sebaceous glands).

It has been stated that the survival period of an untreated homograft is about 2 to 9 days (average 4 days); of the ACTH-treated graft, 16 days; and of the dicumarol-treated graft, 12 days. The buried grafts persisted for as long as 38 days, the limit of usefulness of the tissue-chamber technique. None of these grafts failed. In each case the experiment was terminated because of mechanical factors. Microscopically, we observed the complete absence of thrombosis in these animals. However, the rate at which vascularization proceeded was not equivalent to the rate in autogenous grafts. Capillary budding was marked and capillary loops were seen at the periphery of the graft. This early circulation formed a rapid shunt of blood through these circulatory loops. In these experiments we observed the invasion of the graft by newly formed vascular channels. The evidence points to the fact that the inimical state existing between homoplastic skin and the invasive circulatory response of the host is attenuated or alleviated in part by the graft's nearly complete deprivation of its adnexal structures. This leads to the conjecture that, if homoplastic skin grafts fail because of an immune reaction, the immunity may be invoked by a factor or factors contained in the adnexa or in the excreta of the skin transplant.

The attainment of permanently successful homografts of skin would be a great advance for clinical surgical practice. At present, homotransplants of skin are limited in clinical practice to the temporary coverage of extensive wounds, as in the severely burned child. In such use, it is important that the clinician boldly excise the homograft at an early date (about 4 weeks) after its transplant, before it begins autodissolution. Once that process starts, excessive fibrosis of the resultant granulating bed is the rule. This causes delay in recovery and increases the degree of ultimate deformity. In my own practice, valuable temporary use was made of a skin homotransplant to a hemophiliac who was in critical condition from blood loss. The patient (C. J., New York Hospital case no. 239923) exhibited a large wound of the forearm, extending on its flexor surface from the elbow to the wrist. The wound resulted from surgical incision (done on another service) for the release of a tense blod clot that endangered the circulation of the hand. The homoplastic graft survived, and resulted in complete healing. Its temporary life was recognized and it was excised 40 days afterward. An autogenous skin graft was cut and sutured to the denuded area. Sufficient clotting mechanism was supplied by the hourly administration of 100 cc. of whole blood for 48 hours after operation. Both the autograft and its donor site healed without complication and the patient recovered. This is an example of the strategic temporary value of a homoplastic skin graft. How much advanced surgical care would be, if homografts were permanently successful. Battle casualties as well as injured civilians could quickly be given grafts from tissue banks. Severe and extensive dermatoses no longer would take their toll. The urgency of the need for successful permanent skin transplantation cannot be overemphasized.

The Genetics of Transplantation [1,2]

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Transplantation has been a research tool of cancer workers for more than 50 years. These studies have taught us some things about the biology of cancer and a great deal about the biology of transplantation.

At first, the factors that cause transplanted tumors to grow or fail to grow were largely obscure. Now we can point with confidence to a complex variety of environmental and genetic agencies that influence the degree of their development in the host, though it need hardly be said that the unknown agencies still outnumber the known.

In discussing the genetic agencies, I shall limit myself to genetic factors in the classical sense, *i.e.*, to the genes (chromosomally borne units) of the tumor and of the host. Although I shall discuss only genes and genetic factors, it should be emphasized that I am not denying the existence or importance of environmental influences, or the possibility that self-reproducing cytoplasmic units play a role in the success or failure of transplants.

Successful studies of the genetics of transplantation first became possible with the development of highly inbred strains of mice, produced by repeated brother-times-sister matings. It has been shown both by theory and by experiment that the individuals of any one such inbred strain are, except for occasional mutants, identical. They are like twins derived from a single egg. *The following discussion deals only with work done with inbred strains or their hybrids.*

The first successful explanation of differences in susceptibility and resistance to transplants in terms of Mendel's laws of heredity was made by Little and Tyzzer in a series of papers beginning in 1914 and 1916 (*1, 2*). Since that time the subject has been pursued by Strong, Cloudman, Bittner, Furth, Kaliss, MacDowell, Gorer, Browning, Snell, and others. Because of limitations of time, I shall pass hastily over much of the early work, and because of the specialized genetic techniques involved, I shall

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stress conclusions rather than evidence. The evidence, except for certain recent experiments to which I shall refer, is all on record, and all except the most recent work has been summarized in review papers (*3*, *4*).

Early genetic studies with transplantable tumors led to the formation of certain laws of transplantation, which may be stated as follows:

1) *Tumor isotransplants, i.e.* tumors transplanted within the strain of origin, grow progressively and kill all hosts.

2) *Tumor homoiotransplants, i.e.* tumors transplanted within the species but outside the strain of origin, fail to grow, or grow temporarily and then regress.

3) F_1 hybrids produced by crossing two inbred strains will grow tumors indigenous to either parent strain.

4) Only a fraction of mice of an F_2 generation, or of a backcross produced by mating the F_1 to the resistant parent, will grow tumors from the inbred lines involved. The portion susceptible may be large or small, depending on the tumor and the cross, but rarely exceeds 75 percent for the F_2 or 50 percent for the backcross.

It should be emphasized here that in all genetic studies the criterion of susceptibility is death as a result of progressive growth of the tumor. Cases in which the tumor shows only temporary growth are tabulated as negatives. "Takes" is a term to be avoided in genetic studies, because it implies doubt as to the ultimate outcome of the implant.

The above four laws are based on an extensive body of evidence. There are occasional exceptions, particularly to rule 2. Certain tumors, when transplanted to foreign strains, will grow in a percentage of the hosts, the percentage varying through wide ranges according to the tumor and the host strain. Sarcoma 180 is the classic example of tumors of this type. For most tumors and for most crosses, however, the rules hold with a considerable degree of accuracy.

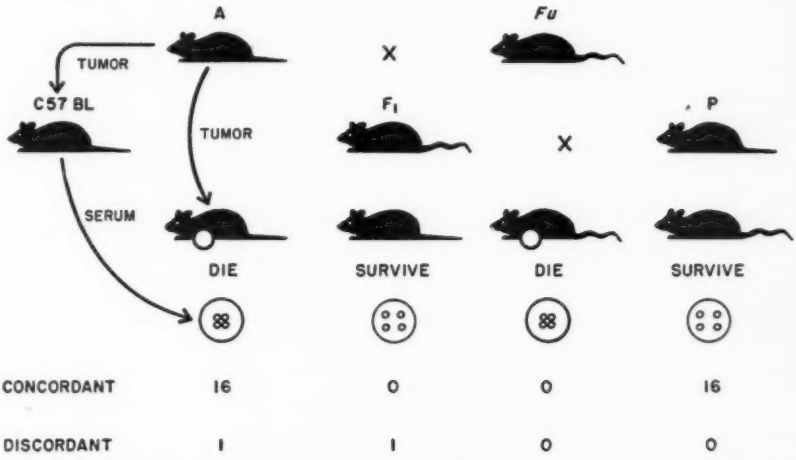
On the basis of these rules, Little and Tyzzer formulated the now generally accepted genetic theory of transplantation. According to this theory susceptibility is due to multiple dominant genes. These genes may be called *histocompatibility genes*. Available data permit only an approximate estimate of the number of histocompatibility genes or genetic loci in the mouse. Certainly the number is not less than 6 or 7; it may be much larger. The same genes that determine susceptibility and resistance to tumor transplants probably also determine susceptibility and resistance to normal tissue transplants (*5*, *6*).

In 1946 a study was started aimed at identifying individual histocompatibility genes in the mouse. By good fortune it was possible to carry out one phase of this study jointly with Dr. Peter Gorer, who had already done important work in England on the relationship of a blood-group gene in the mouse to transplant susceptibility.

Most earlier work had been based simply on inoculation of mice in an appropriate segregating generation with transplantable tumors. Our joint study with Dr. Gorer introduced two new factors: 1) the use of "marker genes"—known genes with visible effects—to detect possible

linkage with histocompatibility genes, and 2) the use of blood typing concomitantly with tumor inoculation. The blood-typing serum was produced in most cases by the inoculation of C57BL mice with a strain A tumor.

These studies revealed a linkage between the gene for fused tail (*Fu*) and a histocompatibility gene which also determined Gorer's red-cell antigen II (7). One typical cross, together with the observed results, is shown in text-figure 1.



TEXT-FIGURE 1.—Association of normal tail, tumor susceptibility, and antigen II in mice from the cross (A × *Fu*) × P, where A and P are two inbred strains, and *Fu* indicates a strain carrying the gene for fused tail. Mice inheriting the fused-tail gene are indicated in the diagram by kinks in their tails. In 32 of the 34 tested mice, those animals that showed red-cell agglutination also showed tumor susceptibility, while those that showed no agglutination showed tumor resistance. These mice are listed as "concordant" since the tests agree in what they indicate as to the presence or absence of an antigen derived from strain A. The 2 "discordant" mice are listed according to the results of the blood test.

Strain A mice were crossed to fused-tail (*Fu*) mice, and the resulting F_1 hybrids crossed to strain P. The offspring of this double cross were classified on the basis of tail structure as fused or normal, tested for blood type with the anti-strain-A-tumor serum, and inoculated with a strain A tumor. In the particular cross shown in text-figure 1 there were 34 mice. It will be seen that nearly all of these fell into 2 groups. Sixteen were fused tail, lacked antigen II (red cells not agglutinated), and were resistant to the tumor. Another 16 were non-fused tail, possessed antigen II (red cells agglutinated), and were susceptible to the tumor. The remaining 2, both non-fused tail, were discordant with respect to results of blood typing and tumor inoculation. One showed agglutination but survived, the other showed no agglutination but died.

Further experiments established the following facts.

1) Susceptibility and resistance in the cross is determined by a histo-

compatibility gene, *H-2*, which also determines the presence or absence of antigen II.

2) Strain A blood type and tumor susceptibility are in this cross one and the same thing. Discordance between the results of a blood test and tumor growth is usually due, not to faulty blood typing, but to an inherent element of inaccuracy in the tumor-inoculation test. There is occasional growth of the tumor in genetically resistant mice, and occasional failures to grow in genetically susceptible mice. Such cases we have called *false positives* and *false negatives* (*8*). The percentage of false positives and false negatives varies greatly with cross and/or tumor. In most crosses, inoculated with tumors of suitable virulence, the erroneously classified animals probably do not exceed 5 percent of the total, and may sometimes be much less than this.

3) The gene for fused tail (*Fu*) and the gene histocompatibility-2 (*H-2*) are separable by crossing over (*8*). A small percentage of fused-tail mice from crosses like that in text-figure 1 are genetically susceptible, as proved by breeding tests, and a few normal mice are genetically resistant. By using strains of fused-tail mice in which crossing over has occurred, it is possible to design crosses in which fused-tail mice regularly die and non-fused-tail mice survive, thus reversing the condition shown in text-figure 1. The percent of crossing over between *Fu* and *H-2* has not yet been accurately determined, but it is probably more than 1 and less than 5 percent. Another dominant mutant gene, brachyury or *T*, is also in this linkage group, and is in most ways preferable to *Fu* as a marker.

We have so far spoken of the gene *H-2* as if it were a single entity. Actually, as is commonly the case with genes in all plants and animals, it exists in several alternative forms. These alternative forms are called alleles.

The methods of identifying these alleles cannot be described here, beyond the remark that they employ the linkage of *H-2* and *Fu*. They have already been published elsewhere (*9*, *10*). The results, however, are easily presented.

Table 1 shows the alleles that have been identified to date, and the stocks in which they occur. Seven major alleles are now recognized, $H\text{-}2^{dk}$, $H\text{-}2^{d}$, $H\text{-}2^{k}$, $H\text{-}2^{b}$, $H\text{-}2^{p}$, $H\text{-}2^{q}$, and $H\text{-}2^{r}$, and one of these, $H\text{-}2^{k}$, has

TABLE 1.—*Distribution of* H-2 *alleles in 19 inbred strains*

$H\text{-}2^{dk}$	$H\text{-}2^{d}$	$H\text{-}2^{k'}$ $H\text{-}2^{k}$	$H\text{-}2^{b}$	$H\text{-}2^{p}$	$H\text{-}2^{q}$	$H\text{-}2^{r}$
A	BALB/c DBA/2 C57BL/6–*H-2*^{d} C57BL/10–*H-2*^{d}	AKR C57BR/a C57BR/cd CBA C3H ST	C57BL/6 C57BL/10 C57L LP 129/Rr	P	DBA/1	RIII/Wy

a minor variant, $H\text{-}2^{k'}$. There is good reason to suppose that this is not the complete list; other alleles will probably be found as additional tests are made.

An interesting feature of the *H-2* alleles is that not one of those so far found is recessive; all express themselves in the heterozygote. This is a parallel to the situation found in the Rh genes and most of the other blood-group genes in man.

Another interesting feature is the occurrence of two components, d and k, a situation that provides a striking parallel to the Rh alleles in man, where three components, C, D and E are known. The two components d and k have so far been found together in only 1 inbred line, strain A. The d component alone has been detected in 4 strains, including BALB/c and DBA/2, while the k component is present alone in 6 strains including AKR and C3H. Eight of the 19 strains so far analyzed lack both components.

These components manifest themselves when F_1 hybrids, one of whose parents is $H\text{-}2^d$ and the other $H\text{-}2^k$, are inoculated with strain A ($H\text{-}2^{dk}/H\text{-}2^{dk}$) tumor 15091a. The result of such inoculation is invariably progressive growth of the tumor. None of the pure parent strains, be they $H\text{-}2^d$ or $H\text{-}2^k$, will grow this tumor except in infrequent instances. It is only when the d and k are brought together in the hybrid, reproducing the dk condition of strain A, that growth occurs. Moreover, F_1 hybrids involving any combination of parents other than $H\text{-}2^d \times H\text{-}2^k$ are preponderantly resistant.

Some typical data are shown in table 2.

TABLE 2.—*Data showing the susceptibility of F_1 mice of the genotype $H\text{-}2^d/H\text{-}2^k$ to strain A ($H\text{-}2^{dk}/H\text{-}2^{dk}$) tumor 15091a. Mice from all other types of crosses show total or partial resistance. + = susceptible (mouse died from tumor). − = resistant.*

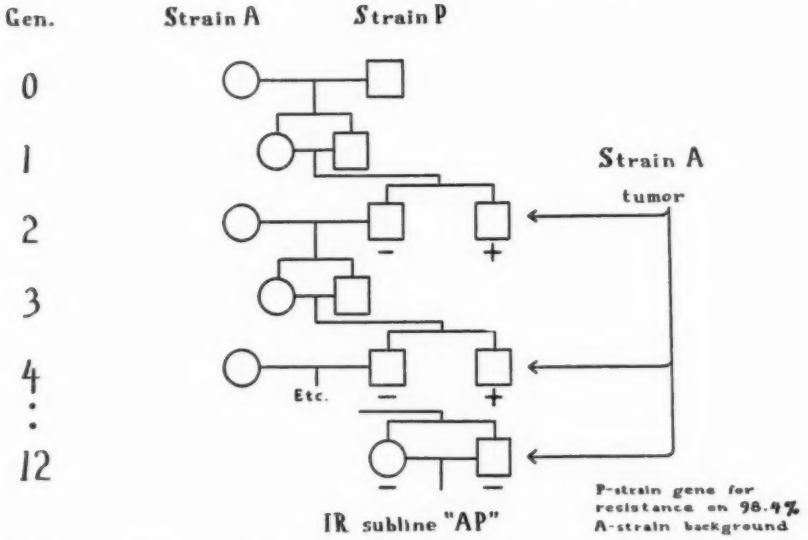
$H\text{-}2^d \times H\text{-}2^k$			Not $H\text{-}2^d \times H\text{-}2^k$		
Cross	+	−	Cross	+	−
BALB/c × AKR	25	0	BALB/c × C57BL/6	1	22
BALB/c × ST	30	0	BALB/c × B10B	0	10
BALB/c × C57BR/cd	15	1	BALB/c × RIII	0	32
BALB/c × C57BR/a	17	0	DBA/1 × C57BR/a	14	12
BALB/c × CBA	25	0	DBA/1 × CBA	5	23
BALB/c × C3H	20	0	ST × C3H	3	16
DBA/2 × AKR	14	0	C57BL/6 × AKR	0	20
B10B × CBA	13	0	P × CBA	2	11

It should be emphasized that all strains were originally classified as carrying $H\text{-}2^d$ or $H\text{-}2^k$ or some other *H-2* allele on the basis of the linkage test. The results shown in table 2 constitute an entirely independent test, which has confirmed in every case the original diagnosis.

The question arises why, when early studies of the genetics of transplantation indicated the existence of a number of genes or genetic loci concerned with resistance, certain recent tests and crosses of a more specific nature seem to reveal only one. We do not yet have the final

answer to this question. However, we have had to think in terms of "strong" and "weak" loci. The *H-2* locus is a "strong" locus. Differences between tumor and host at this one locus will stop the growth of all but the most virulent tumors. Cross-takes between lines with *different* *H-2* alleles are rare.

Cross-takes between lines with the *same H-2* allele are somewhat more common. However, C57BL/6 tumor C1498 tested in 14 LP mice failed to grow progressively in any of the animals. Strains C57BL/6 and LP are both *H-2*^{b}. Many similar cases could be cited. This in itself is evidence for the existence of additional histocompatibility loci.



TEXT-FIGURE 2.—Method for production of isogenic resistant (IR) lines. Strain A is any inbred line, strain P any other line which may or may not be inbred. Mice in each even-numbered generation are inoculated with a strain A tumor and the survivors (indicated by a minus sign) remated to strain A. The resulting isogenic resistant line is designated "AP."

For some time studies have been in progress aimed at analyzing these other loci. These studies are not complete, but some progress has been made.

The method employed is based on the use of what we have called *isogenic resistant*, or IR, lines (*11*). The lines are produced by the succession of crosses shown in text-figure 2.

The purpose of the crosses is the introduction, into an inbred strain, (symbolized in the figure by A) of a foreign histocompatibility gene from a second strain, P. Strain P may or may not be inbred. The procedure is to cross A and P, raise an F_2 generation, inoculate with a strain A tumor, mate the survivors (who must owe their resistance to a gene or genes derived from P) with A, raise a second F_2, inoculate the A tumor,

and so on. In the 12th generation, two resistant individuals are mated together, producing the IR subline "AP."

It can be shown mathematically that, in cases where two histocompatibility genes segregated in the original cross, only one of them will still be present in the IR line in 97.4 percent of all cases. Where more than two genes are segregating initially, the chance of more than one of them being carried along in the crosses until the 12th generation is somewhat greater, but still low.

Aside from the introduced histocompatibility gene, and a certain area of adjacent chromosome carried along with it, the genes of the IR line are 98.4 percent derived from strain A, only 1.6 percent derived from strain P. Essentially then, an IR line, AP, possesses one gene for resistance to transplants isolated from the gene complex of strain P and introduced into the gene complex of strain A. Its resistance to strain A tumors is due, with possible rare exceptions, to this single gene difference.

In setting up the project for the production of isogenic resistant lines, something over 100 lines were started. Some of these have inevitably been lost, so that the number now being carried has been reduced to 74. Slightly more than one-half of these have reached or passed the 12th generation, and testing of these for identification of the introduced histocompatibility gene has been started.

The testing methods involve quite specialized genetic procedures, and only a few details can be given here. The first test applied is a linkage test, with *Fu* or *T* as a marker, to determine if the introduced histocompatibility gene is an *H-2* allele. In tests completed to date, an *H-2* allele has been involved in a majority of the lines. This further emphasizes the importance of this one locus.

The next group of tests involve an entirely different principle. Two strains and one transplantable tumor are involved in each test. These are: 1) an isogenic resistant or IR line, 2) a tumor native to its corresponding pure line, and 3) some third "unknown" line. The procedure is to cross the IR and the "unknown" and to inoculate the F_1 hybrid with the pure-line tumor. Some typical results with one IR line, B^6D^2, derived from an initial cross between strains C57BL/6 and DBA/2, are shown in table 3. The introduced histocompatibility gene distinguishing strain B^6D^2 from strain C57BL/6 has been shown by the linkage test to be an *H-2* allele, but has not been otherwise identified. Since, however, it came from strain DBA/2, the presumption is that it is $H\text{-}2^d$. The tumor used was C1498, native to strain C57BL/6.

It will be seen from table 3, test number 1, that strain C57BL/6 inoculated with its own tumor gave the expected results, 100 percent mortality. Only 2 mice of IR strain B^6D^2 have been tested subsequent to the 12th generation, both being negative (test 2), but since the line is derived from homozygous resistant animals there is little question that it is genetically resistant. Test 3 shows the results of the inoculation of an entirely unrelated "unknown" strain, C3H, with the expected negative results. In test 4, F_1 hybrids from the cross $B^6D^2 \times$ C3H were negative (1 in 20

TABLE 3.—*Tests with IR line B⁶D², identical with C57BL/6 except for the substitution of* H-2ᵈ (*f*) *for* H-2ᵇ. *All mice inoculated with C57BL/6 tumor C1498.*

Test No.	Strain or cross	Genotype	+	−
1	C57BL/6	*H-2ᵇ/H-2ᵇ*	10	0
2	B⁶D²	*H-2ᵈ/H-2ᵈ*	0	2
3	C3H	*H-2ᵏ/H-2ᵏ*	0	50
4	B⁶D² × C3H	*H-2ᵈ/H-2ᵏ*	1	19
5	LP	*H-2ᵇ/H-2ᵇ*	0	14
6	B⁶D² × LP	*H-2ᵈ/H-2ᵇ*	18	0
7	B⁶D² × 129	*H-2ᵈ/H-2ᵇ*	15	0

succumbed). Since these were genetically *H-2ᵈ/H-2ᵏ*, and hence without allele *H-2ᵇ* which is present in the tumor, this result is expected. In test 5, 14 pure strain LP mice were negative. This strain is *H-2ᵇ/H-2ᵇ* (see table 1) and hence so far as the *H-2* locus goes should be susceptible. Its resistance must be attributed to differences between strains C57BL/6 and LP at loci other than *H-2*. In test 6, 18 of 18 mice from the cross B⁶D² × LP succumbed. Here both parent strains are resistant, but the F_1 hybrid is susceptible, a reversal of the result in test 4.

This result is explained as follows. Strain B⁶D² is identical with strain C57BL/6 except for the substitution of gene *H-2ᵈ* for *H-2ᵇ*. All it lacks for the growth of tumor C1498 is this one missing gene. Strain LP has the gene *H-2ᵇ*, but lacks various other genes required by C1498. In the F_1 hybrid the necessary factors are brought together. Strain LP supplies gene *H-2ᵇ*, strain B⁶D² supplies all other necessary genes. The result is susceptibility.

This type of result was predicted when the production of isogenic lines was first proposed (*11*), and the prediction has now been fully confirmed in this and other crosses. One other test is shown in the table (test 7), where B⁶D² was crossed with *H-2ᵇ* line 129. The results are the same as for line LP.

The test just described gives positive results (all mice die) when the pure line (*e.g.*, C57BL/6) and the "unknown" (*e.g.*, LP or 129) have the *same* allele at the locus under test. It gives negative results when the pure line and the unknown (*e.g.*, C3H) have different alleles. In the example in table 3, it was applied to a known situation in order to show that the method is valid. It is now being applied to unknown situations, where it becomes a tool for determining the identity and distribution of histocompatibility genes. We can not go into all the crosses involved, but some of the results to date can be briefly summarized and are presented in table 4.

It will be seen that one new locus, *H-1*, has been definitely identified and another new locus, *H-3*, probably identified. More than half of the 74 isogenic resistant lines are still untested or incompletely tested. It seems reasonable at this stage to presume that when the tests are completed, a thorough analysis will have been achieved of a number of the more important histocompatibility loci in the mouse.

TABLE 4.—*Summary of current status of isogenic resistant (IR) lines*

	Number of lines
Known *H-2*	24
Probable *H-2*	4
Known *H-1*	1
Probable *H-1*	1
Probable *H-3*	1
Known not *H-2* but otherwise untested	5
Probable not *H-2* but otherwise untested	2
Not tested	36
Total	74

Summary and Conclusions

Susceptibility and resistance to transplants in the mouse are determined by multiple dominant genes, called *histocompatibility genes*. The number of such genes or genetic loci can be only very approximately estimated at the present time; there are certainly not less than 6 or 7, the actual number may be much larger.

There is considerable indirect evidence that some genes are "weak" and some "strong." By a weak gene we mean one such that a tumor carrying it will often grow in a strain lacking it. A difference between tumor and host at a strong genetic locus will almost invariably prevent progressive growth of a transplant.

One of the strongest histocompatibility loci is the *H-2* locus. Seven alleles at this locus, $H\text{-}2^{dk}$, $H\text{-}2^{d}$, $H\text{-}2^{k}$, $H\text{-}2^{b}$, $H\text{-}2^{p}$, $H\text{-}2^{q}$, and $H\text{-}2^{r}$, have already been detected; there may be more. The locus has two "components," d and k; heterozygotes of the genotype $H\text{-}2^{d}/H\text{-}2^{k}$ invariably grow strain A ($H\text{-}2^{dk}/H\text{-}2^{dk}$) tumor 15091a.

The *H-2* locus determines a red-cell antigen, as well as susceptibility or resistance to transplants. Whether other histocompatibility genes in the mouse are also blood-group genes is entirely unknown.

The *H-2* locus is closely linked with the genes fused tail (*Fu*) and brachyury or short tail (*T*).

Other histocompatibility loci in the mouse are being studied by means of isogenic resistant (IR) lines. An IR line is defined as one identical with a standard inbred strain except for the introduction of a single gene causing resistance to tumors of this inbred strain. IR lines may be produced at will by appropriate crosses, and 74 are now in the process of production or analysis.

Methods of analysis involve linkage tests, and crosses between different IR and pure lines with inoculation of appropriate tumors. To date one new locus, *H-1*, and a possible third locus, *H-3*, have been identified.

The distribution of *H-2* alleles in 19 inbred lines has been determined. The mapping of *H-1* alleles has been begun.

It should be emphasized that success or failure of transplants is influenced by environmental variables as well as by genetic factors, though the latter are perhaps the better understood.

References

(*1*) LITTLE, C. C.: A possible Mendelian explanation for a type of inheritance apparently non-Mendelian in nature. Science 40: 904–906, 1914.

(*2*) LITTLE, C. C., and TYZZER, E. E.: Further experimental studies on the inheritance of susceptibility to a transplantable tumor, carcinoma (J. W. A.) of the Japanese waltzing mouse. J. Med. Research 33: 393–453, 1916.

(*3*) BITTNER, J. J.: A review of genetic studies on the transplantation of tumours. J. Genetics 31: 471–487, 1935.

(*4*) LITTLE, C. C.: The genetics of tumor transplantation. *In* The Biology of the Laboratory Mouse (G. D. Snell, ed.). Philadelphia, The Blakiston Co., 1941, pp. 279–309.

(*5*) LITTLE, C. C., and JOHNSON, B. W.: The inheritance of susceptibility to implants of splenic tissue in mice. Proc. Soc. Exper. Biol. & Med. 19: 163–167, 1922.

(*6*) KALISS, N., and ROBERTSON, T.: Spleen transplantation relationships among two inbred lines of mice and their F_1 hybrids. (Abstract.) Genetics 28: 78, 1943.

(*7*) GORER, P. A., LYMAN, S., and SNELL, G. D.: Studies on the genetic and antigenic basis of tumor transplantation. Linkage between a histocompatibility gene and "fused" in mice. Proc. Roy. Soc., London, 135: 499–505, 1948.

(*8*) SNELL, G. D.: Preliminary data on crossing over between *H-2* and *Fu*, *Ki* and *T* in the mouse. Heredity 6: 247–254, 1952.

(*9*) SNELL, G. D., and HIGGINS, G. F.: Alleles at the histocompatibility-2 locus in the mouse as determined by tumor transplantation. Genetics 36: 306–310, 1951.

(*10*) SNELL, G. D.: A fifth allele at the histocompatibility-2 locus of the mouse as determined by tumor transplantation. J. Nat. Cancer Inst. 11: 1299–1305, 1951.

(*11*) ———: Methods for the study of histocompatibility genes. J. Genetics 49: 87–108, 1948.

Discussion: The Genetics of Tissue Transplantation

Moderator: Dr. C. C. Little, Roscoe B. Jackson Memorial Laboratory

DR. W. E. HESTON, NATIONAL CANCER INSTITUTE, BETHESDA, MD.: In the session on clinical problems the question was raised regarding the probability that in the human population the recipient would be genetically receptive to an homologous tissue transplant, and how this probability would be affected by choosing the donor and recipient from the same family. In answering the question in very general terms, one should first point out that the human population is a genetically heterogeneous population and that only in the case of identical twins can the genetic similarity between individuals be expected to approach that in the inbred strains of mice. As Dr. Snell pointed out, successful transplantation is dependent upon the recipient possessing certain dominant genes present in the donor and thus in the graft. While the number of these genes is not known, for normal tissues it is presumably large and probably larger than that for neoplastic tissues. Without knowing the actual number of genes concerned, the exact probability that the recipient will be genetically receptive to an homologous graft cannot be determined, but in the general population this probability would be expected to be small, decreasing as the number of genes increases. The probability could be expected to be somewhat higher when the donor and recipient are related than when they are unrelated, and higher when the transplant is made from parent to offspring than when made from offspring to parent.

With respect to some of our own work, it might be emphasized that the recipient need not necessarily be genetically identical with the donor to insure successful transplantation so long as the recipient has those specific dominant genes possessed by the graft and concerned in its successful transplantation. The F_1 hybrid from a cross between two inbred strains possesses all the dominant genes that either parent strain has. Thus, considering the whole complex of genes, since the parent strains are genetically different the F_1 is genetically different from either strain, but the F_1 will successfully accept grafts from either parent strain.

By utilizing these well-known facts one can get two tissues that are genetically different growing in a common host. This situation has been applied to the problem of pulmonary tumors in mice in Dr. Arthur Kirschbaum's laboratory and in our own laboratory, to ascertain whether or not the susceptibility of an animal to the development of pulmonary tumors is inherent in the pulmonary tissue, *i.e.*, whether the primary gene action controlling the development of pulmonary tumors is localized in the pulmonary tissue or is manifest through some general systemic mechanism. Normal lung tissue from a genetically susceptible strain and that from a genetically resistant strain were transplanted into the F_1 hybrid of the two strains. The fact that, following the injection of the hybrid with a carcinogen, tumors frequently developed in the transplanted lung tissue from the susceptible strain but rarely developed in the transplanted lung tissue from the resistant strain, indicated that the primary action of at least most of the genes controlling pulmonary tumor development, by which these strains differed, is localized in the pulmonary tissue.

Dr. Prehn might care to discuss a similar problem he has worked on with mammary tumors in the mouse.

Dr. Richmond T. Prehn, National Cancer Institute, Bethesda, Md.

It has been suggested by Dr. Heston that I discuss our work illustrating the use of the basic genetic laws of transplantation of which we have heard so much this evening.

We have been interested in the problem of the spontaneous mammary tumor in mice. The work we have done is very similar in principle to Dr. Heston's work with

lung tumors. As we have just heard, it is possible to transplant tissues from either parent inbred mouse strain to the F_1 hybrid, and we have utilized this fact in the following way: a strain of mice susceptible to mammary tumors was outcrossed to a tumor-resistant strain. The resulting F_1 hybrids were quite susceptible to spontaneous mammary tumors. Mammary glands were then transplanted from the resistant parent strain to the susceptible F_1 hybrids. F_1 hybrid glands were autotransplanted to serve as controls. All transplantations were done when donor and recipient animals were 3 weeks of age and were still susceptible to administration of the mouse mammary tumor milk agent.

Thus, by the judicious use of the genetic laws of transplantation it has been possible to compare the mammary tumor incidence in glands from mice of high-tumor and low-tumor strains when the glands were in a common physiologic environment. What we have been comparing is the intrinsic glandular susceptibility when all extra-glandular factors, such as hormone levels, etc., have been constant. Results to date have indicated that the glands from the tumor-resistant mouse strain have remained resistant even when growing in the susceptible F_1 recipient, whereas tumors have been observed in transplanted susceptible glands. This finding suggests that resistance or susceptibility to spontaneous mammary tumors is, at least in a large measure, an intrinsic property of the glandular tissue rather than the result of systemic factors.

It is necessary to mention the method of mammary gland transplantation, since previous methods have proved rather unsatisfactory for a variety of reasons. Our nethod is simply to make a full-thickness skin graft (including the panniculus carnosus) containing the gland or glands to be transplanted. The transplanted gland is therefore left in a normal anatomic relationship to surrounding structures, and gives the appearance of being a completely normal and functioning organ. Lactation can be readily observed in such transplanted glands, although we have not yet succeeded in raising litters upon them.

The ease with which full-thickness skin grafting can be accomplished among genetically compatible mice has been utilized in another way. We have heard much tonight about the basic genetic laws of tissue transplantation, and some of the practical research applications of these laws. We have heard very little about the method of gene action or how the gene exerts its influence over tissue transplantation. The same general principles of transplantation can, however, be put to work toward elucidating this problem.

The first question that might be asked is whether genetic differences make the tissue cells of donor and recipient intrinsically incompatible. This problem has been attacked in the following manner.

As we have seen, it is possible to make full-thickness skin grafts from either parent inbred strain to the F_1 hybrid. However, transplantation is usually impossible between the parent inbred strains. We have made skin grafts from one parent strain to the F_1 hybrid, and have then implanted a tumor from the other parent strain into the skin graft. This results in a situation in which the local tissues surrounding the tumor implant are from a strain genetically incompatible with the tumor. The host animal as a whole, however, is susceptible to growth of the tumor. If the tissues are intrinsically incompatible we might expect that the tumor would fail to grow. The results of this type of experiment have indicated that the genetic derivation of the local tissues at the tumor implantation site (*i.e.* skin grafts) does not influence the success or failure of tumor transplantation. The obvious conclusion, that genetic differences do not make tissue cells intrinsically incompatible, is in line with the results of tissue-culture experiments done by other investigators at the National Cancer Institute.

This same type of attack can be made on the problem of secondary or acquired immunity. The phenomenon of concomitant tumor immunity is well known. The first implantation of a tumor exhibiting this phenomenon is successful, but its growth apparently "immunizes" the host so that a second implantation of the same tumor fails.

If such a tumor is placed in the tails of a number of mice of an inbred strain, and the tails are subsequently amputated, the mice will be found "immune" to further implantation of the same tumor. The skins of such immune animals can be grafted to non-

immune mice of the same strain. Conversely, skins of nonimmune animals can be grafted to the immunized mice. Now the stage is set to transplant the same immunizing tumor to these two sets of grafts. Results obtained in such an experiment have indicated that the immunity, whatever its nature, is not fixed in and transplanted with a skin graft. On the other hand, grafts from nonimmune mice to immune animals were consistently resistant to tumor growth. We can therefore conclude that the immunity is dependent upon some type of circulating factor.

I hope that this discussion has illustrated the practical usefulness of present-day knowledge of the genetics of transplantation, and the potential value of continued research of the type described by Dr. Snell.

Dr. George W. Woolley, Sloan-Kettering Institute, New York, N. Y.

I should like to refer to two experimental methods in which genetic procedure and transplantation technique have been used to further biologic knowledge.

Sharp differences exist between strains of mice in their adrenal cortical response to gonadectomy. In strain C57BL there is little adrenal cortical change; in strain DBA, nodular hyperplasia of the adrenal cortex occurs; and in strain CE, adrenal cortical carcinoma invariably occurs. These differences persist in generation after generation, and there are evidently hereditary differences involved. Since these growths may be prevented by hormone replacement, the question arises as to whether the genetic influences operate mainly through systemic or through local adrenal cortical tissue factors to produce the strain differences. A knowledge of the way genetic transplantation laws operate allows a critical experiment to be set up. Dr. Huseby first utilized this technique in relation to the adrenal cortex. More recently we have also utilized it. In our experiments a cross has been made between strain DBA and strain CE. The F_1 hybrids were gonadectomized at birth, adrenalectomized at one month of age, and in some instances received transplants of strain DBA adrenal glands and in some instances transplants of strain CE adrenal glands. The results thus far indicate, as they did in Dr. Huseby's experiment using different strains, that, under the F_1 systemic influence toward adrenal cortical carcinoma formation, strain DBA adrenal glands will only develop toward nodular hyperplasia and strain CE adrenal glands will develop adrenal cortical carcinomas. It is evident that local factors in the adrenal cortical tissue are of great importance in determining these inherited differences in susceptibility to adrenal cortical tumors. An extension of this study will permit determination of the site of genetic differences in hormonal production by these tumors, since strain DBA nodular hyperplasia is associated with a "female-like" hormone and strain CE adrenal cortical carcinomas with a "male-like" hormone.

Another experiment also demonstrates the usefulness of the genetic law of transplantation for securing new information difficult to obtain otherwise. Recently a new dominant-lethal tail mutation has appeared in the mouse that seems to be in part a morphologic mimic of the effect of the gene *T*. The question at hand is how best to secure knowledge of whether or not this new mutation is identical to *T* and to the *T* location on the ninth chromosome. *Ki*, *Fu*, or *T* locations do not seem useful for linkage tests in this case. A linkage test was set up in a standard way, using the *H-2* locus (also a ninth-chromosome locus) and the new tail mutant. Using inoculations of tumor 15091a, it has been possible to secure evidence that the new mutation is not at the well known *T* locus. Although this work must be considered preliminary as far as the actual results are concerned, the method seems to be straightforward and useful.

Dr. P. B. Medawar, University College, London, England

A remark of Dr. Heston's prompts me to raise the question: how often may homografts such as skin homografts in human beings be expected to succeed when donor and recipient are chosen at random? The answer "Never" is perhaps too peremptory,

and Dr. I. J. Good [1] of London has proposed a general solution along the following lines.

Let a be a "histocompatibility" antigen that is present in a fraction p of the population (and accordingly absent from a fraction $1-p$). Let D and R be donor and recipient, respectively, chosen at random. Then the antigen a may be present in any one of the following distributions, to the occurrence of each of which a certain probability may be attached:

	D	*R*	*Probability*
1.....	present.....	absent.....	$p(1-p)$
2.....	present.....	present.....	p^2
3.....	absent.....	present.....	$p(1-p)$
4.....	absent.....	absent.....	$(1-p)^2$

It follows from the definition of "histocompatibility antigen" that only case 1 defines an incompatibility situation, for a cannot act as an antigen in cases 2, 3, and 4. The summed probability of the occurrence of these favorable combinations is simply:

$$p^2 + p(1-p) + (1-p)^2 = 1-p(1-p)$$

Purely for convenience' sake, write $\pi = 2p-1$, so that $\pi=0$ when $p=\frac{1}{2}$. Then it follows by simple algebra that the occurrence of a "favorable" distribution of the antigen a is:

$$\frac{3+\pi^2}{4}$$

Of course, more than one antigen is concerned, and we may consider a whole family $a_1, a_2, a_3, \ldots a_n$ for which the corresponding π values are $\pi_1, \pi_2, \pi_3, \ldots \pi_n$.

The combined chances of getting a "favorable" or compatible combination of the distribution of the n antigens is:

$$\frac{3+\pi_1^2}{4} \times \frac{3-\pi_2^2}{4} \times \frac{3-\pi_3^2}{4} \cdots \times \frac{3-\pi_n^2}{4}$$

or in the conventional shorthand:

$$\prod_{i=1}^{n} \frac{3-\pi_i^2}{4}$$

In Good's words, "It may be optimistic to take $n=20$ but it would be pessimistic to take all the π_i's equal to zero. So it may not be far from the truth" to say that the probability of a successful take is:

$$\left(\frac{3}{4}\right)^{20} = \frac{1}{300} \text{ approximately.}$$

This figure—one permanently surviving graft by luck in 300—is interesting because it represents a most unexpectedly high fraction. There are, however, difficulties in accepting such a figure. Mr. M. F. A. Woodruff has pointed out that n may well be more than 20, and that the percentage of favorable combinations drops very rapidly as n increases. Dr. J. B. S. Haldane challenges Dr. Good's premises on the ground that, if they were true, the percentage of favorable combinations in grafts between parents and offspring should be very much higher than there is any clinical evidence for supposing it to be. It is likely that both these comments will appear in due course in *The Lancet*. In the meantime, I should only deplore any tendency for clinicians to claim successful results from the use of skin homografts on the ground that "mathematics shows it might well be so." Any claim whatsoever that a homograft has survived permanently must be the subject of the most detailed and scrupulous documentation: the *a priori* likelihood of compatibility is, at best, small, and the pitfalls of interpretation are many.

[1] Good, I. J.: Skin banks. Lancet, London, 2: 289, 1952.

Acquired Immunity to the Graft [1]

E. J. Eichwald, *Department of Pathology, University of Utah College of Medicine, Salt Lake City, Utah*

Acquired immunity to transplanted tissues has been discussed widely in relation to spontaneous cancer, tumor transplantation, and transplantation of normal tissues. This presentation is organized accordingly: it deals first with the problem of acquired immunity to spontaneous cancer; then with tumor transplantation; and finally with transplantation of normal tissues.

Investigators of spontaneous cancer have searched intensively for phenomena denoting the presence of acquired immunity to neoplastic cells. They have studied inflammatory reactions around foci of neoplastic cells, and degeneration of neoplastic cells in their original site and after metastatic dissemination. They have looked for antibody-like substances in the serum of cancer patients. They have investigated case histories in which permanent regression of pathologically proved cancers appeared to have taken place. They have attempted immunization for therapeutic purposes by reinjecting modified cancer cells into their original host. These studies have been unsuccessful.

The opposite situation has prevailed in the transplantation of normal tissues. Here one of the chief problems has been that grafts from one individual to another of the same species can be transplanted with some initial success but that after a certain period of time degeneration occurs almost invariably. This observation has suggested strongly the development of acquired immunity by the recipient individual. Attempts to prevent or overcome this resistant state have been unsuccessful.

Immunity to Spontaneous Cancer

Immunotherapy of cancer has been the aim of many investigators, and there are a few who still hold out for it. There is some merit in their arguments. Some cancers, and a slowly increasing number of them, have been demonstrated to follow the introduction of a virus-like agent. If some cancers, and maybe all cancers, are caused by infectious agents, it is only sound to attempt to cure or prevent these diseases by means which

[1] Presented at the Tissue Transplantation Conference, Arden House, Harriman, N. Y., October 8, 1952.

have proved of value in the fight against other infectious diseases, such as smallpox or tetanus.

The hopes for a cancer cure on immunological principles received unjustifiably strong emphasis from the experiments in the field of tumor transplantation. Tumors which were known to grow progressively and kill some hosts, began to grow in others, only to regress after a certain period. Thus they followed a growth curve similar to the temperature curve of typhoid fever or pneumonia. It was also noted as early as the turn of the century that the injection of normal, embryonal, or attenuated neoplastic tissue rendered a host immune to a test inoculation of tumor tissue that would have been fatal without the prior injection of the immunizing agent. At that time not enough was known about genetics and the significance of the host-graft relationship. It was not realized that the chance for a cure of the transplanted cancer was in direct proportion to the height of the genetic barriers separating the graft from the host—species barriers, strain barriers, and the barriers between individuals with closely related genetic constitution.

Immunotherapy of cancer is a subject somewhat in disrepute. We seem to have accepted the dictum that there can be no immunotherapy of cancer. It is considered scarcely in good taste to ask whether there is not something of fundamental importance in the observations of Lumsden (*1*) and others.

Let us consider one of the observations of Lewis and Aptekman (*2*). They studied the growth of a transplantable rat tumor in an inbred strain of rats in which the same tumor had arisen originally. The tumor and the strain therefore had a very similar if not identical genetic constitution, and it grew progressively in 100 percent of the hosts. They strangulated the tumors with a rubber band to choke off the blood supply. The tumors died, became absorbed, and left all hosts immune to reinoculation with the same tumor.

Similar results were obtained by Richter and MacDowell (*3*) with small numbers of leukemic cells which had arisen in the same closely inbred strain; by Goldfeder (*4*) with tumor cells properly attenuated with irradiation; and by Gross (*5*) with small numbers of cells of methylcholanthrene-induced sarcoma, injected intradermally.

How can a host become immune to a tumor of its own? There are three possible explanations: 1) The host and the transplanted tumor are indeed identical or nearly identical genetically, but the neoplastic cells carry an additional antigenic moiety which is specific to it and to neoplastic tissues in general. It is the response to this specific tumor antigen, brought to the fore by the experimental technique of Lewis, Goldfeder, Lumsden, Gross, and MacDowell, which caused the regression of these nearly autologous tumors. This explanation would be most gratifying. It has been the great hope of the cancer immunotherapists. It is the theory which Lumsden attempted to prove, but which today we tend to discredit. The simple observation of Gross that in some animals spon-

taneous tumors arose while the treated tumors regressed should go far in delivering a fatal blow to the theory of tumor-specific antigens.

2) While host and transplanted tumor may have originally had an identical genetic constitution, the circumstances of the experiment resulted either in slow death of tumor tissue, or involved tumor growth under unfavorable local conditions. Antigenically active products were elaborated and the antibody response destroyed the graft. If there should be some basis for this theory, we should search for means of creating or increasing some of this latent antigenicity, by strangulation of the tumor, or by intratumoral injections of antisera or formalin.

3) The third explanation is the most likely and the most discouraging. It goes as follows: the premise of the first 2 explanations is false. Host and tumor were not identical genetically. True, they arose in the same strain and were very similar, but they were not identical. Maybe they were identical at one time; but at the time of the experiment there had developed a genetic dissimilarity, which, if properly magnified, could evoke a strong immune response and destroy the graft. George Snell has reminded us that "any pure stock is slowly but consistently undergoing minor changes in its genetic constitution" (*6*). The observed immunity phenomena would then depend on "the cumulative genetic gap resulting from long continued serial transfer" (*7*). If this is correct, and these experiments were based on the action of iso-antibodies, then "the search for a cure of human cancer based upon these principles is likely to prove a fruitless one" (*8*). We should also be aware that the described phenomena are directed almost exclusively against the establishment of a new tumor, and not against an already established tumor. And we know that right after transfer the life of a graft hangs on a thin thread, even under the most hospitable circumstances. Should we then give up the search for immunotherapy of cancer? I wish to answer this question with a recent statement of Hauschka's: "The reality of autoimmunization in health and disease argues against premature abandonment of the search for neoplastic antigens" (*7*).

Immunity to Transplanted Tumors

1) Induction of immunity.—Some years ago George Snell stated that we should not "regard experiments in tumor immunity as having any but the most remote bearing on the problem of cancer prevention and cancer therapy" (*6*). The reported observations in this field are highly diversified, the number of investigators is legion, and the literature appears endless (*6, 9*).

There is that type of immunity which is evoked by a growing tumor itself: the tumor regresses after a brief period of growth. Then there are the many phenomena which are exhibited by two or more transfers of biological material. Whenever a tumor has regressed in a partially susceptible host, heterologous or homologous, reinoculation of the same tumor will not be successful in most instances. Next, there is that immunity which follows injections of material different from that of the

test tumor. A different tumor or even normal tissue may induce an immune state. Red blood cells will immunize as long as the cells, or major portions of their surface, remain intact (*10*). In the experience of other investigators cell fragments may be sufficient. Dulaney (*11*) has demonstrated that both mitochondria and the microsome fraction suffice. The immune response to whole nuclei is relatively weak.

A great variety of techniques have been used to produce resistant states. The number of injections of antigen has varied from 1 to 10 or more. The doses and the intervals between injections have varied. The injection routes and the techniques of preparation and storage of the antigenic material have varied.

2) Manifestations of immunity.—The results of the immunizing procedures often have not been reproducible. The lack of standardization of experimental procedures, and particularly the failure to control genetic factors has made duplication of experiments difficult. The properties of different host sites have not always been duly considered. Subcutaneous inoculation of a tumor graft into a homologous host in many instances does not give an accurate picture of the susceptibility of a host or its ability to develop a strong immune response. Many grafts fail to grow in this site not because of the immune response of the host to the graft, but for other reasons, such as technical or anatomical circumstances. One of the advantages of the anterior chamber technique is that the factors which may prevent the initial take of a tumor are reduced and standardized (*12*). Almost all grafts are given a chance to grow; if they do not grow progressively, it is attributable to one main biological event, namely the immune response of the host to this particular graft. The anterior chamber technique is a standardizing procedure. It reduces variables.

a. Morphologic aspects.—The most obvious manifestations of the immune effect are morphologic in nature: a tumor gets off to a good start, but after 2 or 3 weeks it ceases to grow and is absorbed; or, a tumor which under ordinary circumstances would have grown, fails to do so if its inoculation is preceded by the injection of antigenic material.

The manifestations of the immune response are not always so precise. The immune response can manifest itself as a decrease in the percentage of takes, as a prolongation of survival of the host, or as a decrease in tumor size.

b. Precipitins, complement fixing, and lymphocytopenia-producing antibodies.—Table 1 shows a variety of antibodies which have been described in association with transplanted tissues, together with the names of investigators associated with them. Although this compilation is impressive, the mechanics of graft regression have not yet been adequately explained.

Complement-fixing antibodies, precipitins, and antibodies which exert a lymphocytopenic effect have been demonstrated in a number of instances, but it is by no means certain that these or other antibodies are responsible for the regression of the graft, or that they can be incriminated in the actual destruction of the foreign cells. They may be mere by-

TABLE 1.—*Antibodies in immune response to transplanted tissues*

Complement-Fixing Antibodies:
- Dmochowski (1938)—(*41*)
- Cruickshank (1941)—(*33*)
- Kidd (1946)—(*13*)

Precipitins:
- Hecht, Sulzberger, and Weil (1943)—(*31*)
- Allgower, Blocker, and Engley (1952)—(*27*)

Lymphocytopenia-Producing Agents:
- Chew and Lawrence (1937)—(*32*)
- Cruickshank (1941)—(*33*)
- Woodruff and Forman (1950)—(*34*)

Hemagglutinins:
- Gorer (1938)—(*42*)
- Lumsden (1938)—(*24*)
- Jay and Kaliss (1949)—(*43*)
- Kaliss and Jay (1950)—(*15*)
- Kaliss and Molomut (1952)—(*44*)

Neutralizing Antibodies:
- Duran-Reynals (1940)—(*45*)
- Kidd (1946)—(*13*)

Neutralizing Antibodies—*Continued*
- Green (1946)—(*18*)
- Burmester (1947)—(*16*)
- Dulaney and Arnesen (1949)—(*11*)
- Law and Malmgren (1951)—(*46*)
- Imagawa, Bittner, and Syverton (1950)—(*19*)
- Werder, Kirschbaum, and Syverton (1950)—(*20*)
- Ambrus *et al.* (1951)—(*17*)
- Ellis and Kidd (1952)—(*23*)
- Stoerk *et al.* (1952)—(*47*)

Cytotoxins in Tissue Culture Experiments:
- Lambert and Hanes (1911)—(*48*)
- Lumsden (1931)—(*1*)
- Phelps (1937)—(*49*)
- Harris (1943)—(*25*)
- Snell and Favata (1951)—(*50*)
- Allgower, Blocker, and Engley (1952)—(*27*)

products which at best indicate that the host is developing some kind of a defense against the graft. Often we fail to demonstrate antibodies of any kind. Occasionally we can demonstrate antibodies, but the graft appears to be little influenced by their presence and continues to grow. In such cases we like to use the argument of the insufficient titer, of the high resistance of the foreign cell, or the low agglutinability of a sample of red blood cells. There is also the possibility that several types of antibody are involved, and that more than one type is necessary to effect graft regression. These different types of antibodies may have different points of attack. One may be associated with the action of inflammatory cells or with vascularization while another may have a direct effect on the cells of the graft by interfering with multiplication. Antiproliferative or "antiblastic" effects of this nature have been discussed by Kidd (*13*), and Medawar's experiments with second-set skin grafts seem to indicate their presence (*14*).

c. Hemagglutinins.—The appearance of hemagglutinins during the development of immune states has been demonstrated by several investigators (table 1). To summarize in a simplified fashion: C57BL mice are resistant to a tumor of strain A mice. Following inoculation of the tumor into the resistant C57BL mice, the latter develop agglutinins which clump the red blood cells of the susceptible strain A. From these experiments it was concluded that tumor cells carry an antigen, characteristic of their strain of origin, and also shared by the red cells of their particular strain. By contrast, the resistant mice lack this antigen and therefore produce agglutinins against the tumor, and against the red cells of the strain of origin, without damage to their own red cells—a situation similar to that in Rh-sensitization. This interpretation implies that susceptibility or resistance to a transplanted tumor is associated with the presence or absence of an antigen in the host—presence in cases of susceptibility and absence in cases of resistance. It has been demonstrated

by Kaliss and Jay (*15*) that these agglutinins develop not because of the presence of carried-over red cells in the graft but clearly because the graft cells themselves carry the antigen. Again, it is debatable whether these agglutinins are responsible for the regression of the graft or whether they represent merely adjuvant or parallel reactions.

d. Neutralizing antibodies.—The demonstration of neutralizing antibodies is a simple and attractive method (table 1). One incubates the material suspected to harbor the antibody with a tumor cell suspension, and injects the material into susceptible hosts. Failure of the tumor to grow in susceptible hosts is an impressive demonstration of the presence of an antibody. However, it is not always satisfactory. When a mouse tumor cell suspension is incubated for one to three hours with the serum of a mouse naturally immune to it, the tumor grows well following inoculation into a susceptible host. The same happens when a tumor cell suspension is incubated with serum of a mouse which has been specifically immunized against the tumor. Exceptions are seen in experiments with the sera of rabbits carrying complement-fixing antibodies against the Brown-Pearce tumor (*13*), and with the sera of immunized avian hosts (*16*). Even when the serum of a naturally immune heterologous host is used an inhibitory effect is barely noticeable. The results are different with the sera of immunized heterologous hosts. These sera readily inhibit or destroy the growth capacity of tumors (*17–20*). The dictum of Leo Loeb's seems to hold, that "immune reactions can be demonstrated the more readily the coarser are the differentials which serve as antigens" (*21*).

Although the sera of immunized mice are free of demonstrable antibody against the homologous tumor used for the immunization, a cytotoxic effect can be shown when one incubates the tumor cells, instead, with minced lymph node from these mice (*22, 23*). By contrast, the lymphoid cells of naturally immune mice do not exert such an effect on homologous tumor cells.

e. Cytotoxins in tissue culture.—Tissue culture methods have been utilized for almost 40 years in the demonstration of cytotoxins. Lumsden was not encouraging with respect to their practicability. He stated that "agglutination tests," by contrast, "have the great advantage that they are easy to perform and to repeat; the results are unmistakable, and can be estimated without special training. Tissue culture experiments require skill and experience" (*24*). Yet, tissue culture methods have been employed to great advantage. They enable us to "separate individual aspects of tissue reactions from the total host response" (*25*). Morgan Harris has demonstrated that rat and mouse cells, in mixed tissue culture, grow side by side and interweave intimately. This will continue for a period of time during which *in vivo* the rat or the mouse would have elaborated a powerful immune response to the foreign cells. To Harris this observation is additional proof that "toxins are not produced by local interaction between graft and foreign body fluids," but, instead, by central agencies. Harris also demonstrated that normal rat serum does not affect the *in vitro*

growth of mouse cancer cells, but the serum of a rat immunized against this particular mouse cancer does exert a lethal effect. This immune serum also destroys the growth in tissue culture of other mouse cancers, from the same or different strain, but it does not destroy cancers from different species. It also destroys cultures of normal mouse cells, but not cultures of normal cells of rats or other foreign species. It appears that the cytotoxins involved are anti-species antibodies. This is in contrast to the findings of Lumsden, who detected fine quantitative differences in the responses of normal and neoplastic tissue. Harris pointedly commented on this by stating that "certain previous workers have tried to twist the action of cytotoxic sera into a specific against tumors"(*26*).

Immunity to Transplanted Normal Tissues

1) *Skin.*—There is a great deal we do not know about the immune response to transplanted tumors. What we know, however, appears great by comparison with the meager information available on the immune response to transplanted normal tissues.

Our knowledge of the fundamental aspects of acquired immunity to transplanted normal tissues with few exceptions is based on morphologic observations. Many homologous skin grafts appear to take initially, but slough after a few weeks. This statement of "sloughing," in most instances, refers to the gross appearance of the graft. Only occasionally have the events prior to sloughing been subjected to microscopic verification. This is quite crucial, since it is known that the microscopic appearance of an apparent "take" can be very disappointing. The epithelium may be completely necrotic, but the graft still appears smooth and vascularized (*27*).

The studies of Medawar have brought our understanding of the sloughing of skin grafts up to a scientific level. He has demonstrated that skin grafts abide by immunological laws: a second skin graft to a rabbit from the same rabbit donor will slough more rapidly than the first. The epithelium of the second graft hardly ever shows mitotic activity (*14*). Medawar has introduced quantitative and semiquantitative methods: he has shown that a large first graft immunizes more effectively than a small one. Medawar has done studies on the nature and specificity of the antigen: the first graft, the immunizing agent, need not be skin; it may be blood, specifically white blood cells, particularly when injected into the dermis of the rabbit (*28*). Medawar also has investigated the mechanics of transmission of the antibody to the skin graft: he has shown that the immune effect is not transmitted to the second graft unless the latter is vascularized (*29*). The immune effect will be absent or weakened when the donors of the first and the second grafts are not identical. This last observation is very impressive: Medawar used rabbits which had been immunized previously with a skin graft. These rabbits received second-set skin transplants, this time to the anterior chamber of the eye but from the same donor. Whenever the skin graft in the eye was not vascularized, the graft survived. Whenever vascularization occurred, the immune

effect was evident by the death of the graft—with one exception. In this one case the graft survived, in spite of rich vascularization of the skin graft in the eye. In this case the second skin graft came from a different donor. In Medawar's own words, "It provided yet another demonstration of the strong donor specificity of the immune reaction."

We have not learned, however, which type of antibody is involved; and we do not know why skin grafts slough. Medawar's experiments indicate that immune phenomena can be elicited with grafted skin and that the fate of skin grafts is influenced by immunization procedures. They do not necessarily explain the sloughing of the first skin graft, although it appears likely that similar events are responsible for this first sloughing.

Early this year a group of workers in Pomerat's laboratory published a significant study on the nature of the immune response to skin grafts (*27*). They were able to produce precipitins and cytotoxins to human skin by immunizing rabbits with human skin suspensions. They failed when they used a homologous source of antigen, namely rabbit skin. It appears that the situation is similar to that in tumor experiments: normal homologous or heterologous serum does not contain demonstrable antibody to the foreign epithelium; nor does immunized homologous serum, but immunized heterologous serum does contain demonstrable antibodies. It takes the coarse contrast between species, potentiated by the immune response, to show the antibodies. The "fine differences either cause insufficient disturbances, or are somehow covered up" (*21*). These results are far from the final aim of the studies: namely, to determine why skin grafts slough. Skin grafts slough not only in heterologous hosts, but also—clinically much more significant—in homologous hosts; and they slough without previous immunization of the host.

It was stated above that circulating antibodies to transplanted mouse tumors had not been detected in the sera of mice immunized against these tumors. By contrast these antibodies had been found to be present in the fixed lymphoid cells of these immunized hosts. A similar situation might pertain with respect to transplanted skin. That does not seem to be the case. Medawar has searched unsuccessfully for cytotoxic antibodies to homologous skin in the lymphoid tissues of immunized rabbits (*30*). Renewed efforts in this direction might prove profitable.

Two groups of investigators have had some success in demonstrating antibodies to homologous transplanted skin. Hecht, Sulzberger and Weil (*31*) worked with 3 groups of rabbits: one group received intramuscular injections of rabbit-skin antigen; the second group received injections of staphylococcus toxin; the third group received both rabbit-skin antigen and staphylococcus toxin; only the animals of the third group developed precipitins to the skin antigen.

The second group of investigators, located in England—Chew and Lawrence (*32*), and later Cruickshank (*33*)—inoculated rat lymph nodes into rabbits and obtained a rat lymphocyte antiserum, which, when reinoculated into rats, depressed the level of circulating lymphocytes.

This lymphocytopenic effect was species-specific, and not dependent on adrenal function: it also occurred following adrenalectomy of the target animal. Woodruff (*34*) did not use the rabbit as an antibody producer, but, instead, another rat of a different strain. He transplanted skin grafts and obtained an antiserum which depressed the lymphocyte level of the donor.

This is where we stand today. To my knowledge, Woodruff's observations on lymphocytopenia-producing antibodies have not been applied to tumor transplantation. Kidd's observations on the presence of cytotoxins in the lymph nodes of tumor-immunized mice have not been applied exhaustively to the transplantation of normal tissues. The extraordinary specificity of the immune response to skin grafts, as observed by Medawar, has not been verified on a larger scale, although its lesson has been applied to corneal transplantation.

2) Cornea.—In the transplantation of portions of the cornea to homologous hosts the problems are similar to those in skin transplantation. One of these is the extent to which immune events influence the outcome of the procedure; another, whether or not corneal grafts ever survive in a biological sense. Considerable light on these two questions has been cast by Maumenee (*35*). He has demonstrated that sensitization of a rabbit recipient, before or after the corneal transfer, almost invariably results in opacity of the graft, provided the antigenic material is taken from the same donor. Opacity is to the ophthalmologist what sloughing is to the surgeon: it indicates failure.

The study of the immune response by Maumenee has supplied information which is apt to cast a shadow on the possibilities of transplantation in general. It has a bearing on true graft survival. The observation that the immune response is not demonstrable in the corneal graft after a period of 6 weeks, indicates either that the grafted cells have adapted themselves to the host, or that they have been replaced by host cells, according to Maumenee. The theory of adaptation requires a great deal of optimism. It is more likely that cellular replacement has occurred. If gradual cellular replacement is the graft's only chance for survival, then corneal transplantation amounts only to a permanent transfer of intercellular substance and the situation resembles that in arterial transplantation. It resembles the insertion of nonliving matter.

3) Endocrine glands.—As early as the turn of this century Halsted (*36*) noted that parathyroid grafts could be autologously transferred with success only when the animal was deficient in parathyroid function. This observation, known as "Halsted's principle," has proved to be of general significance in the transplantation of endocrine glands. Last year Furth (*37*) published a report dealing with the successful transplantation of hyperplastic pituitary tissue to homologous hosts deficient in thyroid function. Halsted's principle somehow modifies the immune response to transplanted tissue. The mechanism of this is not clear. Is the antibody response suppressed? Are the antibodies produced, but held in check by some unknown mechanism? Merwin and Hill (*38, 39*) have shown by the

use of partially thyroidectomized mice with double transplants of thyroid and nonendocrine tissue (harderian gland) that an immune response was produced and that it was not held in check: the thyroid graft persisted while the nonendocrine graft perished. It appears therefore that transplanted endocrine cells are resistant to the immune response when the host is in need of them. What is the mechanism of this resistance? And can this mechanism be utilized by cells of nonendocrine tissues?

Conclusion

Most likely these questions will remain unanswered until more is known about the mechanism of the immune response to transplanted normal tissues. The above sketchy review indicates how little is known. If it is compared with the relative wealth of information on the immune response to transplanted tumors, one might be reminded of some of our friends who like to acquire, without evident purpose, large numbers of mechanical tools such as electrical saws, drills, and lathes, although in practice they are never used. There are only a few investigators who have attempted to apply the accumulated tools. Kaliss and collaborators, for example, have applied the lessons learned in the study of the growth-enhancing or XYZ factor to the transplantation of normal tissues such as skin. The problem is of impressive complexity, and progress has been discouragingly slow. In the past, to quote Medawar, "The plastic surgeon has relied upon the laboratory worker neither for inspiration, principle, nor technical aid." Dempster (*40*), the British surgeon, has said, "The problem is so obscure that the widest research cooperation between experimental surgeon, clinical surgeon, serologist and geneticist will be required for its solution; yet, it is so vital that an organization for the coordination of these specialists would seem opportune, with the aim of achieving successful homotransplantation in man."

References

(*1*) Lumsden, T.: Tumor immunity. Am. J. Cancer 15: 563–640, 1931.

(*2*) Lewis, M. R., and Aptekman, P. M.: Atrophy of tumors caused by strangulation and accompanied by development of tumor immunity in rats. Cancer 5: 411–413, 1952.

(*3*) Richter, M. N., and MacDowell, E. C.: Experiments with mammalian leukemia. Physiol. Rev. 15: 509–524, 1935.

(*4*) Goldfeder, A.: Induced resistance in inbred homozygous rats to a lymphosarcoma autogenous to the strain. Proc. Soc. Exper. Biol. & Med. 59: 104–109, 1945.

(*5*) Gross, L.: Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Research 3: 326–333, 1943.

(*6*) Snell, G. D., Cloudman, A. M., Failor, E., and Douglass, P.: Inhibition and stimulation of tumor homoiotransplants by prior injections of lyophilized tumor tissue. J. Nat. Cancer Inst. 6: 303–316, 1946.

(*7*) Hauschka, T. S.: Immunological aspects of cancer. Cancer Research 12: 615–634, 1952.

(*8*) Barrett, M. K.: The influence of genetic constitution upon the induction of resistance to transplantable mouse tumors. J. Nat. Cancer Inst. 1: 387–393, 1940.

(9) SPENCER, R. R.: Tumor immunity. J. Nat. Cancer Inst. 2: 317–332, 1942.
(10) BARRETT, M. K., HANSEN, W. H., and SPILMAN, B. F.: The nature of the antigen in induced resistance to tumors. Cancer Research 11: 930–936, 1952.
(11) DULANEY, A. D., and ARNESEN, K.: Cytotoxic action of antisera to cell components of normal and leukemic mouse spleens. Proc. Soc. Exper. Biol. & Med. 72: 665–668, 1949.
(12) EICHWALD, E. J., CHANG, H. Y., and LANDA, M.: The significance of the anterior chamber in tumor transplantation. III. Natural resistance and acquired immunity. Cancer Research 12: 490–494, 1952.
(13) KIDD, J. G.: Suppression of growth of Brown-Pearce tumor cells by a specific antibody with consideration of the nature of reacting cell constituent. J. Exper. Med. 83: 227–250, 1946.
(14) MEDAWAR, P. B.: Immunity to homologous grafted skin. I. The suppression of cell division in grafts transplanted to immunized animals. Brit. J. Exper. Path. 27: 9–14, 1946.
(15) KALISS, N., and JAY, G. E., JR.: Do a transplantable tumor and the red blood cells of an inbred strain of mice have an agglutinogen in common? (Abstract.) Cancer Research 10: 227–228, 1950.
(16) BURMESTER, B. R.: The cytotoxic effect of avian lymphoid tumor antiserum. Cancer Research 7: 459–467, 1947.
(17) AMBRUS, J. L., AMBRUS, C. M., HARRISON, J. W. E., and CRAVETZ, H.: Study on the heterotransplantation of Ehrlich carcinoma in relation to antibody formation. Am. J. Pharm. 123: 19–21, 1951.
(18) GREEN, R. G.: Cytotoxic property of mouse cancer antiserum. Proc. Soc. Exper. Biol. & Med. 61: 113–114, 1946.
(19) IMAGAWA, D., BITTNER, J. J., and SYVERTON, J. T.: Cytotoxic studies on mouse mammary cancer cells. (Abstract.) Cancer Research 10: 226–227, 1950.
(20) WERDER, A. A., KIRSCHBAUM, A., and SYVERTON, J. T.: The effects *in vitro* of specific antibodies on the cells of a transplantable mouse leukemia. (Abstract.) Cancer Research 10: 248, 1950.
(21) LOEB, L.: Transplantation and individuality. Physiol. Rev. 10: 547–616, 1930.
(22) KIDD, J. G., and TOOLAN, H. W.: The association of lymphocytes with cancer cells undergoing distinctive necrobiosis in resistant and immune hosts. (Abstract.) Am. J. Path. 26: 672–673, 1950.
(23) ELLIS, J. T., and KIDD, J. G.: Inhibition of Brown-Pearce cancer cells by suspensions of lymph nodes and spleen from immune hosts. (Abstract.) Cancer Research 12: 259, 1952.
(24) LUMSDEN, T.: Agglutination tests in the study of tumor immunity, natural and acquired. Am. J. Cancer 32: 395–417, 1938.
(25) HARRIS, M.: The compatibility of rat and mouse cells in mixed tissue cultures. Anat. Rec. 87: 107–117, 1943.
(26) ———: The role of humoral antagonism in heteroplastic transplantation in mammals. J. Exper. Zool. 93: 131–145, 1943.
(27) ALLGOWER, M., BLOCKER, T. G., JR., and ENGLEY, B. W. D.: Some immunological aspects of auto- and homografts in rabbits by in vivo and in vitro techniques. Plast. & Reconstr. Surg. 9: 1–21, 1952.
(28) MEDAWAR, P. B.: Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. Brit. J. Exper. Path. 27: 15–24, 1946.
(29) ———: Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Brit. J. Exper. Path. 29: 58–69, 1948.
(30) ———: Tests by tissue culture methods on the nature of immunity to transplanted skin. Quart. J. Micr. Sci. 89: 239–252, 1948.
(31) HECHT, R., SULZBERGER, M. B., and WEIL, H.: Studies in sensitization to skin. I. Production of antibodies to skin by means of the synergistic action of homologous skin antigen and staphylococcus toxin. J. Exper. Med. 78: 59–65, 1943.

(*32*) CHEW, W. B., and LAWRENCE, J. S.: Antilymphocytic serum. J. Immunol. 33: 271–278, 1937.

(*33*) CRUICKSHANK, A. H.: Anti-lymphocytic serum. Brit. J. Exper. Path. 22: 126–136, 1941.

(*34*) WOODRUFF, M. F. A., and FORMAN, B.: Evidence for the production of circulating antibodies by homografts of lymphoid tissue and skin. Brit. J. Exper. Path. 31: 306–315, 1950.

(*35*) MAUMENEE, A. E.: The influence of donor-recipient sensitization on corneal grafts. Am. J. Ophth. 34: 142–152, 1951.

(*36*) HALSTED, W. S.: Auto- and isotransplantation, in dogs, of the parathyroid glandules. J. Exper. Med. 11: 175–199, 1909.

(*37*) FURTH, J., and BURNETT, W. T., JR.: Hormone-secreting transplantable neoplasms of the pituitary induced by I^{131}. Proc. Soc. Exper. Biol. & Med. 78: 222–224, 1951.

(*38*) MERWIN, R. M., and HILL, E. L.: The role of vascularization in survival of subcutaneous homografts in mice. (Abstract.) Cancer Research 12: 282, 1952.

(*39*) MERWIN, R. M.: Personal communication.

(*40*) DEMPSTER, W. J.: Problems involved in the homotransplantation of tissues, with particular reference to skin. Brit. M. J. 2: 1041–1049, 1951.

(*41*) DMOCHOWSKI, L.: Uber die serologische Spezifitat der tierischen Tumor- und Eiterantigene. Ztschr. Immunitätsforsch. 93: 311–330, 1938.

(*42*) GORER, P. A.: The antigenic basis of tumour transplantation. J. Path. & Bact. 47: 232–252, 1938.

(*43*) JAY, G. E., JR., and KALISS, N.: A study of antibody production in inbred strains of mice in connection with the growth of transplanted tumors. (Abstract.) Anat. Rec. 105: 536, 1949.

(*44*) KALISS, N., and MOLOMUT, N.: The effect of prior injection of tissue antiserums on the survival of cancer homoiografts in mice. Cancer Research 12: 110–112, 1952.

(*45*) DURAN-REYNALS, F.: Neutralization of tumor viruses by the blood of normal fowls of different ages. Yale J. Biol. & Med. 13: 61–76, 1940.

(*46*) LAW, L. W., and MALMGREN, R. A.: Studies on the "cytotoxic" property of mammary tumor agent antiserum. J. Nat. Cancer Inst. 11: 1259–1268, 1951.

(*47*) STOERK, H., BUDZILOVICH, T., and BIELINSKI, T. C.: Unpublished data.

(*48*) LAMBERT, R. A., and HANES, F. M.: A study of cancer immunity by the method of cultivating tissue outside the body. J. Exper. Med. 13: 505–510, 1911.

(*49*) PHELPS, H. J.: A note on the non-specific action of so-called "anti-cancer" serum. Am. J. Cancer 31: 441–445, 1937.

(*50*) SNELL, A. C., and FAVATA, B. V.: The development of resistance to reinoculation and of circulating cytotoxins in response to heterologous ocular tumor transplantation in the guinea pig. Cancer Research 11: 335–340, 1951.

Discussion: Acquired Immunity to the Graft

Moderator: Dr. J. Kidd, Cornell University Medical College

DR. P. B. MEDAWAR, UNIVERSITY COLLEGE, LONDON, ENGLAND: Dr. Eichwald had time for only the briefest mention of lymphocytes in his lucid and comprehensive address, and I would like to add a few more words about them. As Loeb and Murphy have long since insisted, the infiltration of tissue homografts by lymphocytes is a regular accompaniment to their breakdown. What are they doing? Are they the seats of hypothetical homotoxins, or the vehicles by means of which antibodies are brought to the neighborhood? Or is their presence merely a by-product of the general inflammatory changes that accompany tissue destruction?

There is still no final answer to these questions, because it is hard to think of any *experimentum crucis* that could distinguish between the two possibilities. The quantitative histologic evidence of Darcy [1] tells against the theory of a casual connection between "round-cell" activity and homograft breakdown, but the fact that cortisone can to some degree hold the homograft reaction at bay might be construed as evidence in its favor. But I think that the evidence of *comparative* studies on homograft breakdown is revealing. Very different intensities of inflammatory response and cellular infiltration are to be found in different animals: both are of a savage intensity in the rabbit and the cow, of moderate intensity in the guinea pig, and comparatively mild in mice and men. Yet (forgetting man, for whom the evidence is incomplete) skin homografts live for much the same length of time in all these species; exchanged between animals of the maximal genetic disparity, they survive their transplantation for only 7 to 9 days. This strongly suggests that homograft survival time, intensity of inflammation, etc. are independent variables, and that the intense round-cell infiltration that accompanies homograft breakdown in the rabbit is to be thought of as a serological peculiarity of the species. (*Within* any single species, however, as Dr. Kidd reminds me, intensity of inflammation and shortness of a homograft's life go hand in hand.)

Dr. Nathan Kaliss, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

I would like to submit a few speculations. Up to this point most of our attention has been focused on the reactions of the host to the graft. By implication, the graft has been treated as a passive partner in the host-graft complex. But the graft tissue or organ, as a living structure, must react in a positive manner to the host. Is it not conceivable that the graft also "inflames" to the foreign environment of the host? For example, it has been stated by the clinicians that skin, as a rule, grafts poorly. Leo Loeb has demonstrated that a stronger lymphocytic reaction is observed to skin grafts, even in autologous grafts, than to other organs such as kidney or ovary. Yesterday, Dr. Stark showed us photographs of vascularization reactions around skin homografts in mice. As you recall, the blood vessels of the host grew up to the margin of the graft and then formed a ring around the graft without invading it. May not this be an expression of the inflammatory activity of the graft, in this case leading to its eventual destruction? In the evolution of the living organism, is it not possible that the skin, as the first point of contact with the external environment, has developed the function of inflammation to a very high degree as a specific aspect of its physiology?

[1] DARCY, D. A.: A study of the plasma cell and lymphocytic reaction in rabbit tissue homografts. Philos' Trans. Roy. Soc., s. B. 236: 463-503, 1952.

In this sense, might it not be that the so-called "antigenic simplification" observed in the increased transplantability induced in some tumors is an expression of a lowering of the ability of these grafts to "inflame"?

Dr. Blair O. Rogers, Presbyterian Hospital,[1] *New York, N. Y.*

I would like to draw attention to the fact that Hecht and Sulzberger's paper cannot be considered conclusive evidence of precipitating antibodies directed against the *skin* used in their experiments, because of the possibility that this skin was contaminated with the normal staphylococci often present in the air or environment of any laboratory experiment not conducted under purely sterile conditions. These authors mentioned this fact in a footnote attached to their paper. The obvious conclusion is reached. The antibody they demonstrated was probably directed against a specific staphylococcus contamination rather than against the specific hypothetical skin antigen *per se*.

In our experimental work with humans in the past year, I feel that we have demonstrated a possible new finding in the phenomenon of the human skin homograft. I offer these findings only as a preliminary report, and with a great deal of hesitancy, because we are not yet sure whether these results can be said to be the consistent findings in every case of skin homografting. We have found that during the entire clinical course of a skin homograft there is a *circulatory* as well as a *tissue* eosinophilia. This eosinophilia describes an arc or a curve, reaching a peak of intensity that returns to normal eosinophil levels only when the last vestiges of the skin homograft's dermal pad have completely disappeared by gross examination. Tissue counts of these eosinophils were made from biopsy specimens taken every 4 to 5 days during the course of the survival and ultimate sloughing of the homografts. These counts were made at the interphase between the host tissue and homograft. Without going into detail, because this is to be reported in a paper soon to be released, several hypotheses are suggested. The eosinophilia may be only a response to foreign protein *per se* (Biggart has shown this experimentally in animals). Then again, if the skin homograft may be considered to be a parasite living in the host's environment, trying to change this environment to suit itself or creating conditions to which the host reacts antagonistically, this eosinophilia may actually be the human's response to a parasite. It is well known that eosinophilia occurs with most cases of parasitism in humans.

However, we more strongly favor the hypothesis that the eosinophilia is telltale evidence of an "ill-defined allergic response" taking place in the tissues, which is somewhat similar to the response seen in skin sensitivity reactions with ulceration as described by allergists, or as seen in the tuberculin reaction.

Because there is a high content of histamine involved in the latter two phenomena, and because the eosinophil has been proposed as the cell that carries or manufactures histamine, we might think of the eosinophil as a "causative" cell that needs to make its appearance at the site of the homograft to initiate the sloughing reaction, which it does by furnishing histamine to the tissues around such a homograft. I need not go into the effect of histamine on the end capillaries, with their resultant loss through a gangrenous stagnation and thrombosis. This would differentiate the eosinophil's role from that proposed by Squire, namely that the eosinophil is only concerned with mopping up the products of tissue damage. This also does not rule out the role of the lymphocyte in skin homografting, which may be supplying antibody to the site of the homograft at the same time that the eosinophil is performing its own specific function.

The question might be raised as to the reason for the failure of ACTH to prolong the survival time of skin homografts if, as is well known, ACTH suppresses the eosinophil count (as evidenced by a circulatory eosinopenia in animals or patients given ACTH). If the eosinophil has anything whatever to do with the onset of the sloughing phenomenon, as I have hypothesized, it may be that mere circulatory evidence of an eosinopenia in ACTH-treated animals or humans does not rule out the fact that the suppressed eosinophils may be forced into their tissue and marrow reserve stations, only

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to make their appearance when the mechanism of a homograft's incompatibility demands them at a certain stage in the "ill-defined allergic response." This is suggested by recent clinical and experimental reports of a rise in circulatory eosinophils at the time when the first changes characteristic of destruction of the graft make their appearance, *despite* the continued suppression of these eosinophils by ACTH therapy. Then again, the eosinophils suppressed by ACTH therapy may be of a delicately different morphologic and cytologic configuration than the eosinophils that respond to homografting procedures. In this respect, the eosinophils examined in our counts and biopsies were uncommonly large, very heavily granular, and in the "secretory phase."

We would also like to report that we can substantiate Professor Medawar's second-set "immunity" phenomena in our controlled experiments with full-thickness skin homografts in *humans*; namely, second-set homografts taken from the same donor and applied to the same host undergo a more rapid gangrenous degeneration than the first-set homografts.

Dr. Glenn H. Algire, National Cancer Institute, Bethesda, Md.

Interstrain homografts of a mammary adenocarcinoma were studied in transparent chambers. As with grafts between mice of the same inbred strain, initial vascularization was accomplished by the graft. Temporary growth occurred for approximately 6 to 8 days. Tumor cells survived no longer than 11 days. Vascular breakdown was concurrent with, but not primarily causative of the homograft destruction. Second homografts were made into mice in which a first implant had regressed. There was an early inflammatory reaction and no initial vascularization of the tumor. Tumor cells survived no longer than 6 days.

In agreement with Tyzzer (1916) and Gibson and Medawar (1943), no evidence was found of the existence of natural immunity to the first graft. Evidence was obtained of the development of resistance in the course of exposure to the first, as indicated by the more rapid destruction and failure of initial vascularization of the second homograft.

Dr. Ruth Merwin, National Cancer Institute, Bethesda, Md.

Our observations may be of interest in relation to the mechanism of the initiation of resistance and the mechanism of destruction.

Dr. Eichwald raised the question as to whether a tissue such as thyroid in a thyroidectomized host survived because it was not vulnerable or because it did not initiate immunity. Using Dr. Algire's transparent-chamber technique, it was found that when implants of both thyroid gland and harderian gland from newborn mice of one strain were present in a semithyroidectomized host of another strain, the thyroid survived but the harderian gland disintegrated, suggesting that the thyroid survived in spite of resistance in the host.

Dr. Eichwald mentioned Medawar's finding that nonvascularized homografts survived in an immune host. These implants were made into the anterior chamber of the eye of rabbits. At a subcutaneous site, we have found that nonvascularized homografts of thyroid or harderian gland—unlike Medawar's anterior chamber implants—disintegrated in resistant mice. Other observations of interest here are that while vascularized implants disintegrated, nonvascularized implants survived indefinitely if no vascularized homografts were present in the same host. They survived because no immunity was initiated, for when additional harderian gland implants were made at intervals up to 9 months after the inoculation of the original graft, the new as well as the old harderian gland always survived. Although these nonvascularized implants survived indefinitely they remained vulnerable, for if a new implant that became vascularized was placed in a chamber with a nonvascularized implant that had already been in the chamber for 9 months, the old as well as the new implant disintegrated. After 9 months in a mouse of a foreign strain the nonvascularized implants

were still potentially antigenic, as shown by their disintegration if they became vascularized following reimplantation into the same foreign strain.

Dr. Anna Goldfeder, The City of New York Department of Hospitals, and New York University, New York, N. Y.

In 1905 Ehrlich made the classical observations that when a tumor graft failed to produce a tumor in the animal host, or when an established, actively growing tumor regressed spontaneously, the animal became resistant or "immune" to subsequent viable tumor grafts. Since then, attempts have been made to induce a resistant state experimentally.

Several investigators have demonstrated that tumor grafts attenuated by either moderate heat, formalin, or X radiation rendered animals resistant to subsequent viable tumor grafts. Thus, using hybrid Swiss albino mice and tumor grafts of Sarcoma 180 irradiated with critical doses of X ray, I was able to produce a resistant state in about 100 percent of the treated mice.[1]

Attempts were later made to transfer this resistance or "immunity" against Sarcoma 180 to normal Swiss mice, by injecting fresh or dried lyophilized blood, serum, plasma, or suspensions of various organs, all prepared from the immune mice. However, all such attempts failed to render the animals "immune" to Sarcoma 180.

On the contrary, a rather stimulating effect was noted on the rate of growth of Sarcoma 180 following injections of the lyophilized tissue material into the mice. This was evidenced by the shortening of the latent period (time between the implantation of the tumor grafts and occurrence of a tumor of a measurable size), and by the increase in size of the actively growing tumors compared with those in control mice. Since these observations seemed rather paradoxical, they were not mentioned in the publication.[1]

Further experiments along these lines were carried out on an inbred strain of rats (Bagg strain) with an autogenous tumor.[2] Both were obtained from the late Dr. Halsey J. Bagg. The rats had been bred in his laboratory by brother × sister mating for more than 15 years. The tumor, which arose in a rat of this strain and was diagnosed by the late Dr. James Ewing as a reticulum-cell-type lymphosarcoma, had reached its 75th generation of transfer; the tumor takes were almost 100 percent, and spontaneous regression rarely occurred.

This type of tumor proved to be rather radiosensitive. Thus, tumor grafts irradiated with doses ranging from 2,400 to 2,600 r failed to produce tumors, but rendered a significant percent of the rats resistant to subsequent viable tumor grafts.

However, grafts of autologous fetal skin of the same inbred line of rats failed to induce resistance against this tumor.[3] It has occasionally been observed that rats in which either a control (nonirradiated) tumor graft failed to produce a tumor, or in which an established control tumor regressed spontaneously, also became resistant to viable tumor grafts. Therefore, I am rather inclined to believe that in this case the tumor, and not the rats, underwent a spontaneous genetic change. The fact that the autologous embryonic skin failed to produce resistance in the adult rats supports this view.

Experiments were made on inbred C57BL mice with mammary carcinoma 755, which arose in a mouse of this inbred line. A dose of 4,500 r prevented the tumor grafts from producing a tumor.[4] However, the mice in which the irradiated tumor grafts failed to produce a tumor remained susceptible to further viable tumor implants.

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[1] GOLDFEDER, A.: Relation between radiation effects and cell viability as indicated by induced resistance to transplanted tumors. Radiology 39: 426–431, 1942.

[2] GOLDFEDER, A.: Induced resistance in inbred homozygous rats to a lymphosarcoma autogenous to the strain. Proc. Soc. Exper. Biol. & Med. 59: 104–109, 1945.

[3] GOLDFEDER, A.: Failure of homozygous embryo skin to prevent growth of autogenous tumor-grafts in the rat. Proc. Soc. Exper. Biol. & Med. 60: 338–340, 1945.

[4] GOLDFEDER, A.: Further studies on the relation between radiation effects, cell viability, and induced resistance to malignant growth. Effects of roentgen rays on Bagg-Jackson mouse carcinoma 755 irradiated in vitro and in situ. Radiology 44: 283–292, 1945.

To investigate further the possibility of inducing "immunity" to autogenous tumors, experiments were extended to two highly inbred lines of mice, both bearing the milk agent and implanted with autogenous mammary adenocarcinomas. These are the C3H high-cancer line obtained from Dr. J. J. Bittner, and the DBA high-cancer line obtained from Dr. T. S. Hauschka. The autologous tumor grafts, attenuated with threshold doses of X radiation, failed to induce resistance in the lines of mice from which the tumors originated, whereas mice of sublines of these strains were rendered resistant to viable grafts of the respective mammary tumors.[5, 6]

A significant number of the highly inbred C3H and DBA mice in which either the irradiated tumor grafts failed to produce a tumor, or in which established, actively growing tumors regressed following X radiation, later produced spontaneous mammary tumors.

It was of interest to note a rather stimulating effect on subsequent tumor growth occurring in the C3H and DBA mice in which autologous irradiated tumor grafts, previously injected, had failed to produce tumors. As in the previous experiments with the hybrid Swiss strain of mice, the evidence was the shortening of the latent period. For example, the average latent period of the mammary adenocarcinoma autogenous to the Bittner C3H mice was reduced from about 16–18 days to 10–14 days in the C3H mice previously injected with irradiated tumor grafts.[6] This stimulating effect, however, should be further investigated on a more statistical basis.

To complete the brief account of the experience in this phase of investigation, I would also like to mention the experiments carried out on two highly inbred strains of rats, the August strain and the A × C strain. Grafts of the mammary adenocarcinoma autogenous to the August strain of rats, attenuated with critical doses of X ray (about 5,000 r), as well as X-ray attenuated grafts of the fibrosarcoma autogenous to the A × C rats, failed to render the rats of the respective lines resistant to viable tumor grafts of the respective autologous tumors, subsequently implanted.

In summary, experiments were carried out with three highly inbred lines of mice (C57BL, C3H, and DBA) and on two inbred lines of rats (A × C and August), and with tumors that originated in these lines, respectively. Grafts of the autogenous tumors attenuated with critical doses of X radiation failed to render the animals "immune" or resistant to subsequent viable tumor grafts. It seems that X radiation did not alter the genetic make-up of the tumor tissues so as to render them "antigenic" or foreign toward the host from which they originally arose. Moreover, the possibility of immunizing the host against its own tumor is very questionable.

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[5] GOLDFEDER, A.: Further studies on the relation between radiation effects, cell viability, and induced resistance to malignant growth. Anomalous radiosensitivities of analogous mouse mammary adenocarcinomas. Radiology 54: 93–115, 1950.

[6] GOLDFEDER, A., and INOUYE, F.: Factors concerning radiosensitivity and "immunizing ability" of mouse mammary tumors. (Abstract.) Cancer Research 12: 265, 1952.

Methods of Conditioning the Graft in Tumor Transplantation [1, 2]

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The word "conditioning" implies an oriented change induced by controlled factors. This definition is not entirely applicable to the present topic, for transplantable tissue is not a uniformly reactive system. A few cubic millimeters of solid tumor or of ascitic fluid constitute heterogeneous competitive complexes of millions of cells, amenable—but unpredictable in response—to experimental interference. Conditioning the graft should therefore be viewed as a population problem. The chemical or genotypic environment encountered by a neoplasm in serial passage might be planned so that growth characteristics are altered. Are the observed shifts in morphology, resistance, virulence, and histocompatibility instances of "induced adaptation," or are they brought about by selective mechanisms? Do these changes occur suddenly, in stepwise fashion, or gradually; do they persist or can they be reversed?

The Neoplastic Population

In a panmictic Mendelian population mating at random, genetic equilibria obey the Hardy-Weinberg law: the relative frequencies of each gene allele in the total pool remain constant from generation to generation, unless the alleles endow their carriers with different degrees of viability or reproductive capacity.

The checks and balances in a population of cancer cells are somewhat more subtle. Growth of a tumor graft by purely vegetative division does not result in a tissue in which all the malignant cells are identical. Mutants and viable heteroploid variants accumulate with time. Such a community attains rather labile non-Mendelian equilibria between its clonal components and is a competitive continuum transcending the lifespans of successive hosts by many thousand cell generations. Serial passage introduces an element of discontinuity; the race between competing cell types is called back to the starting line at regular intervals, exhausted substrates are replenished, and toxic or immunologic hurdles

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are intermittently removed. There also is no penalty against metabolic short-cuts, and new contestants may enter the field at any point.

A species holding its ground against other forms in an open environment constantly eliminates mutants that have lost important metabolic functions. The cancer cell, on the other hand, is parasitic within the genotype from which it has itself been derived. This encourages variability with impunity. Lwoff (*1*) has emphasized that parasites have in many cases lost quite a number of functions and that such "losses" may have selective advantages in a medium such as the host environment which contains enough ready-made metabolites. The advantage of not performing certain syntheses may "lead to an acceleration of some other essential growth reactions—that is to an increase in growth rate." A tumor graft is thus potentially a very complex population against which diverse selective pressures can operate and which might even be "conditioned" simply by choice of the proper selective agent.

The mosaic concept of the neoplastic cell community gains support from many facts, some commonplace and others not yet generally appreciated. The attainment of so-called autonomy (*2*), stepwise losses of histocompatibility genes (*3*), the spontaneous transitions from carcinoma through mixed tumor to sarcoma, and the replacement of differentiation by anaplasticity are all suggestive of natural selection from among a multiplicity of cellular variants. Tissue cultures of melanoma, hepatoma, and thyroid tumors, when reimplanted into mice, have resulted in sarcomas (*4*). Even the fatal step from normal to malignant has repeatedly occurred *in vitro* (*5*, *6*). An amelanotic subline has been derived from melanoma S91 by selective transfer (*7*, *8*); and a spontaneous change in nuclear structure from spherical to lobated has been recorded for a distinct subline of 6C3HED lymphosarcoma (*9*).

The wide range of cell size in several ascites tumors (*10*), the multinuclear and giant cells often seen, as well as the fluctuations of chromosome numbers (*11–14*), are proof of a variegated genetic constitution, as are the values of DNA per cell arrived at by chemical and spectrophotometric means (*10*, *15*). There is reason to believe that even cells with somewhat less than the normal diploid chromosome constitution, or aneuploid types unbalanced in the opposite direction, have continued viability.

"Conditioning" such an array of reactive entities from without is not always a question of judicious treatment. Almost any experimental interference (by radiation, drugs, hormones, high and low temperature, antisera, storage, or ligation) is apt to favor one cell type above another, and thereby modifies the graft. The following account will be confined to a few of the more clear-cut conditioning phenomena, namely, the results of exposure to metabolic antagonists, passage through hybrid genotypes, transformation from the solid to the ascites growth form, and the possible dependence of certain conditioning responses on the chromosome ploidy of tumors. Attempts to modify the behavior of a neoplasm by microisolation of clonal sublines will also be described.

Fluctuation Test

Technical and theoretical guidance in the analysis of oncologic population problems has come from advances in bacterial genetics. Ten years ago microbiology was faced with the need for a critical decision between two alternatives in explaining the origin of bacterial variants resistant to phage, antibiotics, and other unfavorable factors:

1) Does the unfavorable factor induce a refractory state in the formerly susceptible organism? Or—

2) Has resistance arisen as a chance mutation, and did the hostile environment therefore play merely a selective role?

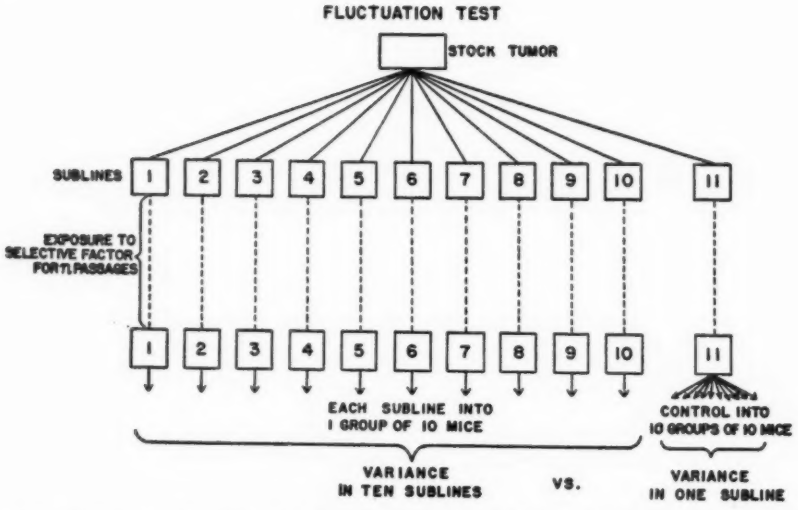
An ingenious statistical method known as the "fluctuation test" was developed by Luria and Delbrück (*16*) to distinguish between these two possibilities. If mutation to resistance is not caused by exposure, the number of resistant cells per culture should vary greatly, depending on how long ago the mutation had occurred; and variance of the number of resistant individuals in different cultures should be larger than the mean number of such cells per culture. If, on the other hand, resistance is an adaptation to exposure, variance should be about as large as the mean. Since, in most experiments of this type, variance was much greater than the mean, it has been concluded that mutation to resistance is independent of exposure.

Law (*17*) has employed the fluctuation test in answering the question of whether or not the resistance of leukemic cells to folic-acid antagonists was caused by exposure. Had the resistant type arisen by adaptive interaction with the antagonist (A-methopterin), there should have been no significant difference between the fluctuation of data for fifteen independent sublines and a single control subline, all derived from the same sensitive tumor. However, variance was twenty times greater in the independent series than in the single subline group, increases in resistance during serial transfer were stepwise, and resistance in the absence of A-methopterin was stable. These results support the mutation-selection mechanism in preference to a conditioning influence of the antagonist on the leukemic cells.

The usefulness of the fluctuation test (text-fig. 1) is, of course, not limited to resistance phenomena; it is adaptable to a wide variety of questions pertaining to the origin of shifts in morphologic, biochemical, functional, and immunogenetic characteristics of transplantable tumors.

Induced Changes in Histocompatibility

Some transplantable tumors which have arisen in inbred stocks, and have been propagated only in the stock of origin, can be conditioned to undergo abrupt changes in their histocompatibility by passing them temporarily through a first-generation hybrid between the susceptible strain of origin and any resistant strain. This important observation was first recorded by Barrett and Deringer (*18*) for a C3H mammary carcinoma. Two groups of resistant backcross hybrids were tested for susceptibility;



TEXT-FIGURE 1.—Diagram showing design of a typical fluctuation test. Donor of stock tumor has received smallest inoculum necessary for take.

one series received tumor grafts directly from C3H donors, the other was implanted with tumor that had resided in (C3H × BALB/c)F_1 mice for one or more passages. Transfer via F_1 was followed by a threefold increase in the backcross takes expected on genetic grounds. The data obtained after direct home-strain passage were compatible with a 3 or 4 *H*-factor ratio; indirect passage gave a 2-factor ratio (expectation 25%).

These experiments have been extended (*19*) to determine whether the altered transplantability was permanently fixed, or whether it depended on maintenance of the tumor in heterozygous hosts and could be changed back to its original level by growing the "adapted" tissue for several generations again in the strain of origin. Barrett and Deringer found that returning the modified tumor to prolonged serial passage in C3H mice could not reverse the acquired decrease in specificity, and that the change had become permanent after a single transfer through C3H × BALB/c. The increase in transplantability applied only to backcross takes and was not accompanied by a generalized trend toward genetic indifference. Statistically, it appeared to be a loss of one or two histocompatibility genes.

Following Barrett's conditioning method, I induced significant shifts in the antigenicity of two further tumors (table 1). The TA3 mammary adenocarcinoma (A/He origin) was tested as an ascites in (A/He × C3H/St) × C3H/St backcross mice; the DBA lymphoma (DBA/2 origin) was inoculated both as an intraperitoneal ascites and as a solid subcutaneous growth into animals of the constitution (DBA/2 × C3H/He) × $C3H_1$/He. Altogether 1,307 mice received these tumors via direct home-strain transfer and indirect F_1 passage. Again, the latter type of transfer somehow

modified the percentage of backcross takes. This change was not haphazard, but usually proceeded from a good fit for a given gene number to a value fitting another gene requirement. However, in three cases out of four an increase rather than a decrease in specificity was recorded. The DBA lymphoma ascites, tested in two series, gave a significantly increased *H*-factor requirement in one experiment and an inconclusive result in the other, while the two control values were almost identical. The stability of tumors following direct passage, and unpredictable instability after transient residence in a heterozygote would appear to detract from the usefulness of genetic data obtained for tumors after they have been routinely propagated in F_1 hybrids.

TABLE 1.—*Comparison of backcross takes resulting in death after uninterrupted passage of mouse tumors in the strains of origin and after one transfer through F_1 between susceptible and resistant genotypes*

Tumor	Direct transfer (*percent deaths in BC*)	Transfer via F_1 (*percent deaths in BC*)	D/SE	Hypothetical shift in antigenic factors
C3HBA carcinoma (solid)*...	7.8 ± 1.6	24.0 ± 3.2	4.5	4 or 3→2
TA3 carcinoma (ascites).....	28.0 ± 3.2	11.2 ± 3.1	3.7	2→3
DBA lymphoma (ascites) Series I.....	24.0 ± 2.1	11.6 ± 3.2	3.3	2→3
DBA lymphoma (ascites) Series II.....	22.0 ± 2.1	34.0 ± 4.7	2.3	2→?
DBA lymphoma (solid).....	5.1 ± 2.2	0.9 ± 1.0	1.8	4 or 5→?

* The data for C3HBA are those of Barrett and Deringer, 1950, who tested 456 mice. The results for TA3 carcinoma and DBA lymphoma are based on 1,307 BC animals inoculated with these tumors.

It is of interest that the solid form of the DBA lymphoma after direct passage required 4 or 5 *H*-factors for successful subcutaneous growth in backcross mice, while the DBA lymphoma ascites needed only 2 genes for progressive intraperitoneal growth. This raises the question whether transplantation site, or stroma, or physical state of tumor (solid *vs.* suspended) can affect antigenicity; for when the solid DBA lymphoma was reconverted into an ascites tumor (both having grown exclusively in the home strain), it immediately returned to its original 2-gene requirement.

A further complication is introduced by estrogenic stimulation, to which lymphoid tumors are particularly susceptible (*20*). Under our experimental conditions, males injected with the DBA lymphoma ascites survived longer than females. The tumor killed a significantly larger percentage of backcross and F_2 females than males of corresponding genotype and age, all inoculated from the same ascites pool with a dosage that has been invariably lethal for all pure DBA mice of both sexes. In table 2 the results for DBA lymphoma Series I and II have been combined and analyzed for sex-differences in response. The effect of indirect transfer, shown in table 1, is canceled out by this rearrangement of the data and a significant influence of sex becomes apparent. The difference

TABLE 2.—*Influence of donor genotype on the sex-difference in percentage of DBA lymphoma takes*

Genotype of donor	Total mice in BC test	Percent takes in BC hosts			Total mice in F_2 test	Percent takes in F_2 hosts		
		♀♀	♂♂	D/SE		♀♀	♂♂	D/SE
DBA/2	100♀ 100♂	35.0 ± 4.8	11.0 ± 3.1	4.4	107♀ 100♂	61.8 ± 4.6	48.0 ± 5.0	2.1
F_1 (DBA/2 × C3H$_f$/He)	100♀ 103♂	27.0 ± 4.4	18.5 ± 3.8	1.5	100♀ 100♂	61.0 ± 4.9	62.0 ± 4.9	0.1

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between takes in males and females was, however, significant only after inoculation from DBA/2 donors. Indirect transfer via F_1 mice narrowed or suppressed the sex-difference in susceptibility.

The conditioning of histocompatibility relationships between graft and host incident to a brief growth period in a heterozygote is obviously a very intricate process of "induced adaptation." This designation does not imply that a genuine adaptive mechanism is postulated (*21–23*). One may speculate that the recessive *h*-loci or partially dominant *H*-alleles in the heterozygous host permit the elaboration of small amounts of antibodies which interact with the corresponding antigens or even genes in the graft. No matter how many antigenic loci are carried in the chromosomes of an individual cancer cell, they are probably neutral in the inbred strain of origin; but some of them could be deleterious, others beneficial in the presence of "hybrid substances" (*24*). Selective factors in the F_1 environment might thus favor variants possessing more or fewer *H*-genes than the modal cell type, and the population equilibrium could drift either toward decreased or increased specificity. The irreversibility of the change in transplantation potential following F_1 passage, the full manifestation of the change after a single such passage, and the good agreement between the modified take percentages and Mendelian ratios all suggest that "immunologic selection" may be at work in the genotypic conditioning of transplantability. The fluctuation test and microisolation procedures (to be discussed below) might be advantageously applied to this question.

Transformation of Solid Tumors Into Ascites

Free tumor cells occur frequently in peritoneal and pleural effusions associated with visceral malignancies. The well known Ehrlich ascites tumor of the mouse arose spontaneously from a solid growth, as did the Yoshida sarcoma of the rat (*25*). The latter was found as a thick milky suspension of cells in the abdominal cavity of a rat with an induced scrotal tumor that had infiltrated the retroperitoneal tissue; it has been transplanted as a typical ascites tumor for more than 300 serial passages.

In the present context, a question of primary concern is the nature of the change from the attached to the suspended growth form. Has the ascites descended from mutant types with a special capacity for continued life and reproduction in a fluid medium? Can any solid tumor be conditioned so that it becomes transmissible as a "nearly pure culture" of cells in peritoneal exudate?

The technique for transforming a solid graft into an ascites (*26*, *27*) consists simply of intraperitoneal inoculation of tissue mince in saline. This results in numerous nodules attached to the mesentery, and invasive growth sometimes accompanied by an effusion. It is a matter of chance whether malignant cells are exfoliated into the serous liquid and whether any of them can multiply there without stromal support and vascular contact. Repeated intraperitoneal subinoculation of exudate may transmit the tumor and the pure ascites state is eventually attained. Fluid

transfer can be interrupted by subcutaneous injection and, due to spatial confinement, the tumor again assumes the solid form exhibiting all its original histologic characteristics. On the surface, this makes the ascitic transformation appear like an adaptational change. However, upon reinjecting the solid tumor—even after several subcutaneous passages—once more into the peritoneal cavity, an ascites now develops immediately (*10*). The initial slow conditioning process, therefore, should be attributed to inadvertent selective transfer rather than to training in the adaptive sense.

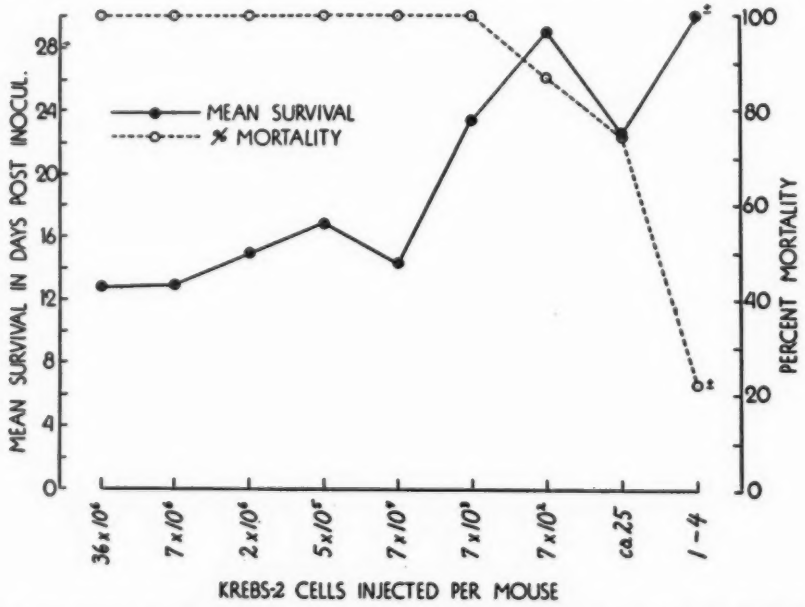
Not all tumors respond equally to this treatment. Klein's representative series of 28 neoplasms, including lymphomas, carcinomas, sarcomas, and melanomas (*10*, *28*), gave results falling into three categories:

a) Some tumors transformed into ascites immediately; this pertained especially to lymphomas, which are derived from tissue accustomed to life in a fluid medium under normal conditions.

b) Several sarcomas and carcinomas conformed only after prolonged passage of the exudate.

c) A third group (notably osteosarcomas, melanomas, and hepatomas) was refractory to the change and grew only as solid intraperitoneal masses.

Rapid growth and an advanced degree of anaplasticity appear to be the primary prerequisites for transformation into a typical ascites. During the earlier transfer generations it is important to inject large cell



TEXT-FIGURE 2.—Survival time and mortality of Swiss mice in relation to Krebs-2 cell dosage.

dosages (about 20 × 10^6 cells). In later generations the inocula may be reduced to very small numbers of cells, as evident from the dosage-mortality curve for the Krebs 2 carcinoma (text-fig. 2). In the most characteristic ascites, such as the DBA lymphoma and the Krebs 2 (figs. 1–3), individual cells are freely suspended and are capable of multiplying in the liquid. Klein (*10*) has shown for the Ehrlich tumor that after four days about five times the number of cells originally injected can be recovered from the ascitic fluid, at a time when solid infiltration is just beginning.

Formation of floating clusters of cells in preference to free suspension appears to be a constant and characteristic feature of some carcinomas and hepatomas; thus the TA3 mammary adenocarcinoma (A/He origin) has continued to form suspended cell complexes of adenomatous arrangement for 67 transfer generations, and this tendency could not be overcome by differential centrifugation of the exudate prior to transfer (Hauschka, unpublished data). Similarly, in Yoshida's ascites hepatoma of the rat (*25*), the cells are never separated from one another, as in the case of the Yoshida sarcoma, but form conglomerates of various sizes and shapes tightly covered with an endothelial layer of Kupffer-cell derivation.

The wide variety of response to the ascites conditioning process, ranging from highly susceptible to completely refractory, indicates that only certain types of cells within any one neoplastic population can survive and grow in the fluid medium of the peritoneal exudate. Experimental conditioning, therefore, does not affect the population as a whole, but simply provides the opportunity for a shift in equilibrium.

Growth Potential as a Function of Chromosome Ploidy

All neoplasms that have been analyzed for chromosome-number distribution were found to be complex heteroploids or chromosomal mosaics (*29*) with a distinct diploid or tetraploid mode (*11–14*). The cells with the most balanced genomes seem to function as "stem-cells" in the propagation of the malignant tissue. There are, however, enough viable cells with aberrant aneuploid chromosome numbers to provide a constant source of genetic inequalities. How are these differences reflected during passage of a graft through foreign genotypes? When fourteen tumors of known ploidy were examined for transplantability in various host strains, a consistent relationship between chromosome constitution and histocompatibility became evident (*30, 31*). All tumors with diploid chromosome modality were strain-specific; the heteroploid neoplasms exhibited varying degrees of genetic indifference with regard to their host requirements (table 3). This principle appeared to be independent of the tumor's origin, transplantation history, virulence, and method of induction. For example, the diploid and tetraploid lymphomas #1 and #2, both induced in strain A females by irradiation with the same dosage, and carried through comparable numbers of serial passages in strain A mice (*32*), behaved as follows: Both tumors were 100 percent lethal in the home strain. The tetraploid lymphoma #1 was easily "conditioned" to serial passage in

TABLE 3.—*Inverse relationship between chromosome ploidy and compatibility in fourteen transplantable tumors*

Tumor and strain of origin	Modal ploidy	Foreign host strains allowing progressive (lethal) tumor growth
DBA lymphoma (DBA/2)	2n	none of six tested
6C3HED lymphoma (C3H/St)	2n	none
TA3 carcinoma (A/He)	2n	none*
A-lymphoma 2 (A/He)	2n	none*
Yoshida sarcoma (Jap. stock rat)	2n	none
15091a carcinoma (A/St)	4n	DBA (31%), all F_1 of genotype *H-2*^{d}/*H-2*^{k}
A-lymphoma 1 (A/He)	4n	DBA (93%), (DBA/2 × C3H)F_1 (100%)
MC1M sarcoma (C3H)	sub-4n	A/He (73%), A/St (71%), Swiss (73%)
S91 melanoma (DBA/Jax)	4n	BALB/c (80–100%)
HS1 sarcoma (Swiss)	4n	C3H$_f$/He, A/He, C57BL (up to 100%)
Sarcoma 180 (Crocker mouse)	4n	many strains (60–100%)
Sarcoma 37 (stock mouse)	4n	many strains (usually 100%)
Ehrlich carcinoma (stock mouse)	4n	nine strains tested (100%)
Krebs 2 carcinoma (Swiss)	4n	nine strains tested (100%)

*"None" applies only to pure strains. The strain A tumors were able to grow in DBA × C3H hybrids of genotype *H-2*^{d}/*H-2*^{k}, which is equivalent to *H-2*^{dk} in the strain A mice.

DBA/2 where it now causes a high percentage of deaths. The diploid lymphoma, although the more virulent, was nevertheless unable to grow in any of seven foreign genotypes tested, including DBA/2. Both tumors "took" in hybrids between C3H and DBA/2, which are genetically *H-2*^{d}/*H-2*^{k} and carry one dose each of the *d* and *k* components discovered by Snell (*33*) in the histocompatibility-2 locus of strain A.

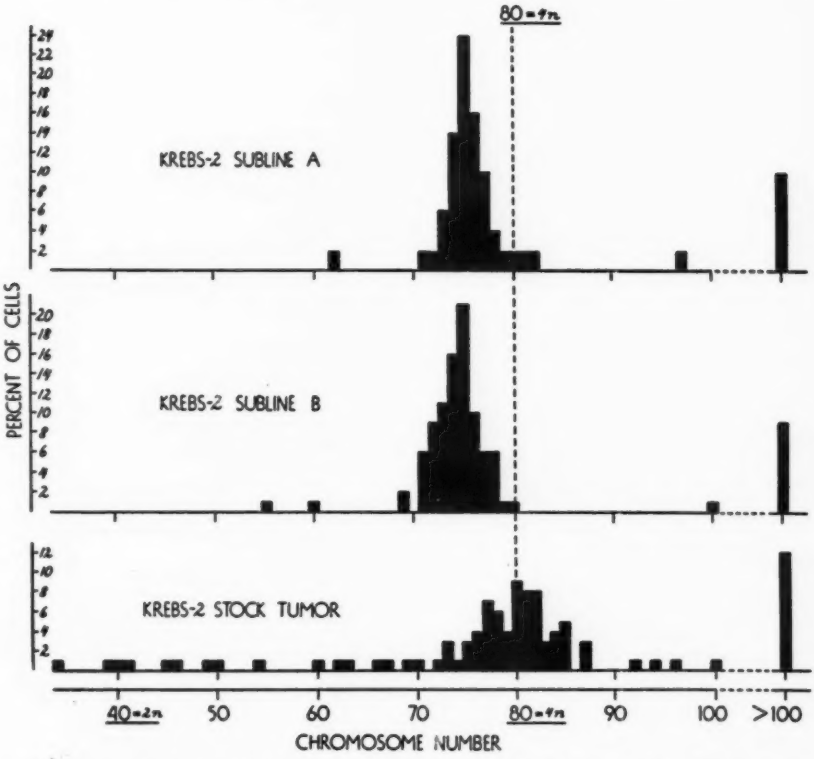
The wider transplantability of the tetraploid category was interpreted as resulting from disturbances in genic balance consequent to polyploidization. In a polyploid cell more than two loci are available for each gene, and allelic *H*-factors can function in dosages other than those to which they are restricted in a diploid, so that antigenic end-products no longer elicit protective antibody in adequate titer. Spontaneous and experimentally conditioned loss of specificity may, thus, be viewed as "immunologic selection" of the least antigenic types from among the viable chromosomal variants in a graft.

This question was approached experimentally through exposure of host-specific diploid tumors to cold shock and colchicine with the aim of producing polyploidy, and thereby changing host-specificity. Although a pronounced c-mitotic effect was apparent shortly after treatment, doubling could not be stabilized and the tumors reverted to their characteristic diploid condition.

Another and perhaps more critical approach is microisolation of single cells, followed by establishment of clonal sublines and comparison of their behavior. Of the few methods for single-cell transfer *in vitro* (*34*) or *in vivo*, the technique recently described by Ishibashi appears to be the most useful for this purpose, since it gave more than 50 percent takes with the Yoshida sarcoma ascites (*35*). The Japanese technique, with minor

modifications, was tried in a preliminary experiment with the Krebs 2 mouse carcinoma (figs. 2–4). This ascites is favorable for a selection experiment because of its heterogeneity. Chromosome numbers range from 34 to over 500; the cells are large, and growth is rapid.

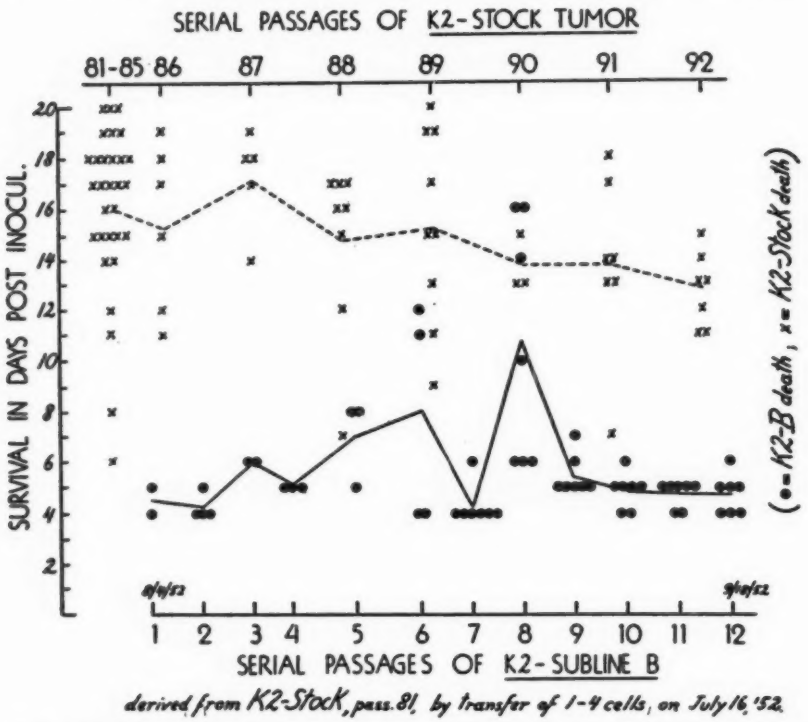
Sixteen infant Swiss mice, anesthetized by cold, each received an injection of one to four cells delivered intraperitoneally from a capillary pipette attached to an Emerson micromanipulator. In two of these mice, typical ascites developed 19 and 21 days after inoculation, and were established in serial passage as sublines A and B. It is not known whether these lines were derived from a single cell, but it is certain that not more than 4 cells were injected. A striking difference between the two sublines and the Krebs 2 stock tumor became apparent from exact chromosome counts (text-fig. 3). Distribution was considerably narrowed, and the modal value, formerly at 80 chromosomes, shifted to 75. A minor mode caused by quadrupling of the diploid set occurred at about 150 for the



TEXT-FIGURE 3.—Distribution of chromosome numbers in the Krebs-2 mouse carcinoma ascites and in two sublines of this tumor. Sublines A and B were derived from generation 81 of the K-2 stock by inoculation of 1 to 4 cells into infant Swiss mice. 250 well-spread metaphases were counted with a rare error not exceeding ±1 chromosome.

sublines and at 160 for the stock tumor. The variations slightly to the right and left of 75 can be ascribed to nondisjunction and lagging, which would occur even if a single cell had been injected (*36*).

It is highly interesting that both sublines, expecially B, were more virulent than the stock tumor (text-fig. 4, table 4). The cell types



TEXT-FIGURE 4.—Comparison of the virulence of Krebs-2 stock tumor and subline B, expressed as survival time. Abscissae for the two tumors are scaled according to days between successive transfers covering the period from August 4 to September 18, 1952.

TABLE 4.—*Comparison of survival time of mice inoculated with Krebs-2 ascites and two sublines derived from the stock tumor by isolation of 1 to 4 cells*

Tumor line	Mortality (*percent*)	Distribution of mortality	Mean survival (*days*)	Standard deviation	D/SE
K-2 stock..	100	multi-modal	12. 91 ± 0. 33	5. 92	Stock and A 0.4
Subline A..	100	bimodal (5 and 15 days)	9. 58 ± 0. 72	5. 09	A and B 0.4
Subline B..	100	unimodal (5 days)	5. 90 ± 0. 41	2. 92	Stock and B 4.2

isolated from generation 81 were apparently held in check by other elements in the parental population, and in spite of their greater virulence were unable to displace competitive variants.

The success of the Japanese workers, and our own initial results, promise that with sufficient perseverance true clonal sublines will soon be isolated from tumors of inbred strains.[3] The cytologic, antigenic, and metabolic properties of such material should reveal the range and nature of differences within any one neoplastic population. This will permit a more critical appraisal of the mechanisms by which tumor grafts respond to experimental conditioning.

References

(*1*) LWOFF, A.: Some problems connected with spontaneous biochemical mutations in bacteria. Cold Spring Harbor Symp., Quant. Biol. 11: 139–155, 1946.

(*2*) GREENE, H. S. N.: A conception of tumor autonomy based on transplantation studies: A review. Cancer Research 11: 899–903, 1951.

(*3*) STRONG, L. C.: Changes in the reaction potential of a transplantable tumor. J. Exper. Med. 43: 713–724, 1926.

(*4*) SANFORD, K. K., LIKELY, G. D., EVANS, V. J., MACKEY, C. J., and EARLE, W. R.: The production of sarcomas from cultured tissues of hepatoma, melanoma, and thyroid tumors. J. Nat. Cancer Inst. 12: 1057–1077, 1952.

(*5*) GEY, G. O.: Cytological and cultural observations on transplantable rat sarcomata produced by the inoculation of altered normal cells maintained in continuous culture. (Abstract.) Cancer Research 1: 737, 1941.

(*6*) SANFORD, K. K., EARLE, W. R., SHELTON, E., SCHILLING, E. L., DUCHESNE, E. M., LIKELY, G. D., and BECKER, M. M.: Production of malignancy *in vitro*. XII. Further transformations of mouse fibroblasts to sarcomatous cells. J. Nat. Cancer Inst. 11: 351–375, 1950.

(*7*) HESSELBACH, M. L.: Control of melanization of S91 tumors by selective transfer, and biochemical studies of the tumors produced. Third Conference on Biology of Normal and Atypical Pigment Cell Growth. New York Acad. Sc., 1952.

(*8*) LOUSTALOT, P., ALGIRE, G. H., LEGALLAIS, F. Y., and ANDERSON, B. F.: Growth and histopathology of melanotic and amelanotic derivatives of the Cloudman melanoma S91. J. Nat. Cancer Inst. 12: 1079–1117, 1952.

(*9*) LEVAN, A., and HAUSCHKA, T. S.: Nuclear fragmentation—a normal feature of the mitotic cycle of lymphosarcoma cells. Hereditas 39: 137–148, 153.

(*10*) KLEIN, G.: Comparative studies of mouse tumors with respect to their capacity for growth as "ascites tumors" and their average nucleic acid content per cell. Exper. Cell Research 2: 518–573, 1951.

(*11*) HAUSCHKA, T. S., and LEVAN, A.: Characterization of five ascites tumors with respect to chromosome ploidy. (Abstract.) Anat. Rec. 111: 467, 1951.

(*12*) LEVAN, A., and HAUSCHKA, T. S.: Chromosome numbers of three mouse ascites tumours. Hereditas 38: 251–255, 1952.

(*13*) MAKINO, S.: A cytological study of the Yoshida sarcoma, an ascites tumor of white rats. Chromosoma 4: 649–674, 1952.

[3] Since these pilot experiments were reported at the Arden House Conference, critical inoculation of single cells gave successful results with two mouse ascites tumors, the Ehrlich carcinoma and the Krebs 2 anaplastic carcinoma. Working with a 1:100,000 dilution of ascites in a medium consisting of 30 parts special mouse Ringer's solution to 1 part cell-free peritoneal exudate, and using an Emerson micromanipulator at a magnification of 200 ×, I have recovered 19 tumor clones from 212 Swiss mice of both sexes within 16 to 25 days after intraperitoneal injection of a single Ehrlich or Krebs 2 tumor cell per mouse. The clones were cytologically more uniform and more virulent than the two stock tumors from which they were isolated. The controversial concept of *stem-cells*, from which each tumor propagates itself, appears to be a reality borne out by the preferential recovery of certain aneuploid chromosome numbers (mostly hypo-tetraploid), representing the most viable portion of the population curve.

(14) KOLLER, P. C.: Abnormal mitosis in tumours. Brit. J. Cancer 1: 38–47, 1947.
(15) LEUCHTENBERGER, C., KLEIN, G., and KLEIN, E.: The estimation of nucleic acids in individual isolated nuclei of ascites tumors by ultraviolet microspectrophotometry and its comparison with the chemical analysis. Cancer Research 12: 480–483, 1952.
(16) LURIA, S. E., and DELBRÜCK, M.: Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28: 491–511, 1943.
(17) LAW, L. W.: Origin of the resistance of leukemic cells to folic acid antagonists. Nature 169: 628–629, 1952.
(18) BARRETT, M. K., and DERINGER, M. K.: An induced adaptation in a transplantable tumor of mice. J. Nat. Cancer Inst. 11: 51–59, 1950.
(19) ———: Induced adaptation in a tumor: Permanence of the change. J. Nat. Cancer Inst. 12: 1011–1017, 1952.
(20) KAPLAN, H. S., and BROWN, M. B.: Inhibition by testosterone of radiation-induced lymphoid tumor development in intact and castrated adult male mice. Cancer Research 11: 706–708, 1951.
(21) BARRETT, M. K.: Some immunogenetic influences upon transplanted tumors. Cancer Research 12: 535–542, 1952.
(22) HAUSCHKA, T. S.: A discussion of some influences on the transplantation and growth of tumors. Proc. 2d Nat. Cancer Congress. Cincinnati, 1952. In press.
(23) ———: Immunologic aspects of cancer: A review. Cancer Research 12: 615–633, 1952.
(24) IRWIN, M. R.: Immunogenetics. *In* Advances in Genetics (M. Demerec, ed.). New York, Academic Press, 1947, pp. 133–159.
(25) YOSHIDA, T.: Studies on an ascites (reticulo-endothelial cell?) sarcoma of the rat. J. Nat. Cancer Inst. 12: 947–969, 1952.
(26) KLEIN, G., and KLEIN, E.: The transformation of a solid transplantable mouse carcinoma into an "ascites tumor." Cancer Research 11: 466–469, 1951.
(27) GOLDIE, H., and FELIX, M.: Growth characteristics of free tumor cells transferred serially in the peritoneal fluid of the mouse. Cancer Research 11: 73–80, 1951.
(28) KLEIN, G.: Development of a spectrum of ascites tumors. Exper. Cell Research 2: 291–294, 1951.
(29) BEATTY, R. A.: Heteroploidy in mammals. Animal Breeding Abstr. 19: 283–292, 1951.
(30) HAUSCHKA, T. S.: Relationship between chromosome ploidy and histocompatibility in mouse ascites tumors. (Abstract.) Cancer Research 12: 269, 1952.
(31) HAUSCHKA, T. S., and LEVAN, A.: Inverse relationship between chromosome ploidy and host-specificity in sixteen transplantable tumors. Exper. Cell Research 4: 457–467, 1953.
(32) SHELTON, E.: Origin and behavior of two transplantable lymphomas induced by X radiation. J. Nat. Cancer Inst. 12: 1203–1223, 1952.
(33) SNELL, G. D.: A fifth allele at the histocompatibility-2 locus of the mouse as determined by tumor transplantation. J. Nat. Cancer Inst. 11: 1299–1305, 1951.
(34) SANFORD, K. K., EARLE, W. R., and LIKELY, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246, 1948.
(35) ISHIBASHI, K.: Studies on the number of cells necessary for the transplantation of Yoshida sarcoma. Gann 41: 1–14, 1950.
(36) SATO, H.: On the chromosomes of Yoshida sarcoma. Studies with tumor cells proliferated in the peritoneal cavity of the rat transplanted with a single cell. Gann 43: 1–16, 1952.

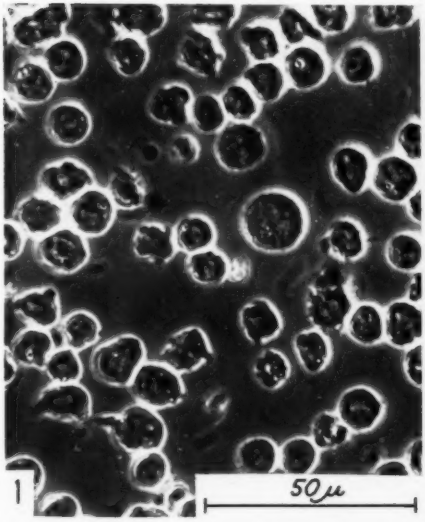
PLATE

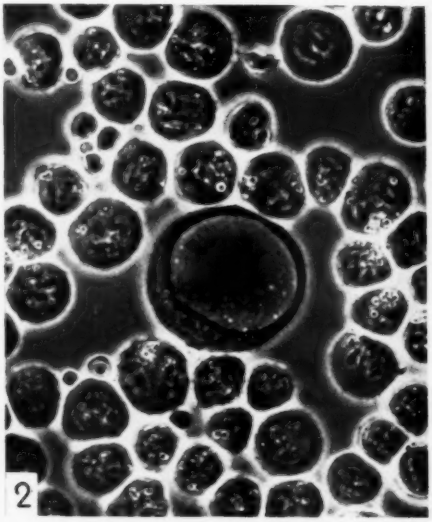
PLATE 49

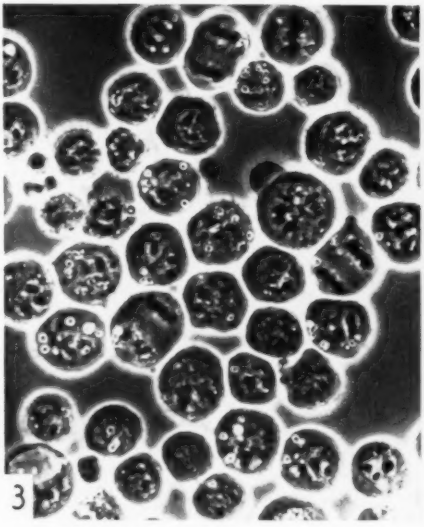
FIGURE 1.—Living cells of the diploid DBA lymphoma ascites, as seen with a dark-phase high-dry objective.

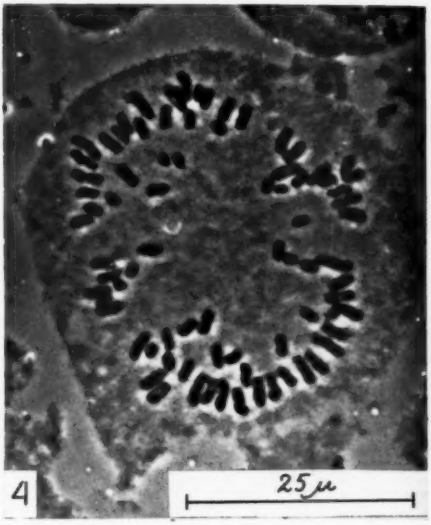
FIGURES 2 AND 3.—Two fields of the tetraploid Krebs-2 carcinoma ascites photographed with a dark-phase high-dry objective. (Same magnification as figure 1.)

FIGURE 4.—Tetraploid Krebs-2 metaphase from an aceto-orcein smear, photographed with a dark-phase immersion objective. (This figure is reproduced from *Hereditas* 38: 252, 1952 by permission of the editors.)









Hauschka 739

273201—53——27

Discussion: Methods of Conditioning the Graft

Moderator: Dr. M. K. Barrett, National Cancer Institute

DR. VIRGINIA J. EVANS, NATIONAL CANCER INSTITUTE, BETHESDA, MD.: For the past two years Dr. Earle and I have been studying the cultivation of human skin, in collaboration with the Naval Medical School's Tissue Bank and the Naval Medical Research Institute. Two problems have been paramount, namely: the possible alteration of skin homograft response through use of tissue-culture techniques, and the growth of large, long-term cultures containing a mixed population of fibroblasts and squamous epithelial cells. We have been able to maintain together in culture predominant cellular elements of the skin for periods in excess of 18 months. One of the early difficulties encountered was the apparent overgrowth of the fibroblast population with a resultant relative diminution in the number of squamous epithelial cells present. In an effort to control this overgrowth we have attempted to find differential methods for selectively inhibiting the fibroblast. Cortisone, cortisone acetate, desoxycorticosterone, desoxycorticosterone acetate, vitamin E, and an incubation temperature of 32° C. have all given uniformly dismal results in permanently inhibiting mesenchymal elements. At the suggestion of Dr. Dan H. Campbell, of the California Institute of Technology, it was decided to try specific inhibition of fibroblasts by using an antiserum with antifibroblastic potential. Therefore, we have had prepared two antisera, one prepared from large cultures and totaling 25 grams of human skin element, which appeared morphologically to be predominantly fibroblastic, and one from human placenta. The former has demonstrated cytotoxic activity for all elements of human skin, leaving the impression that this material probably contained both fibroblastic and epithelial elements in its original antigenic state. However, the antiplacental serum seems to be more specific and we are now able to control fibroblastic proliferation to a limited degree in our mixed cultures of skin using this antiserum. It is hoped that in the near future we can make available large cultures of epithelial cells.

*Dr. C. Andrew Bassett, National Naval Medical Center, Bethesda, Md.**

I should like to emphasize two points and raise a question. The first of these points is that large mixed cultures of both human epidermal and human dermal cellular elements are now available for grafting purposes. The second is that these can be successfully grafted to granulating surfaces and will take initially. The permanency and functional adequacy of these cells is currently being investigated. The question I should like to propose for consideration is this: Is there a possibility that "cultured cells" are more embryonal than the original donor cell and in such a state will persist for longer periods following grafting? Drs. Gaillard, Gey, and Greene have suggested that normal embryonic tissue may have altered homograft response.

Dr. Hamilton Baxter, Royal Victoria Hospital, Montreal, Canada

I greatly enjoyed Dr. Hauschka's extensive review of various methods of conditioning the graft, since it has given a detailed cross section of previous investigation. Our studies on homografting at the Royal Victoria Hospital in Montreal have been carried out exclusively on man.[1] An attempt was made to obtain permanent survival of skin homografts in man by treating them as follows: 1) By storage at 4° C., 0° C., −20°

* *Present address:* New York Orthopaedic Hospital, Columbia-Presbyterian Medical Center, New York 32, N. Y.

[1] BAXTER, H., and ENTIN, M. A.: Experimental and clinical studies of reduced temperatures in injury and repair in man. Plast. & Reconstruct. Surg. 3: 303-334, 1948.

C., and −78° C., for varying periods of time. 2) By repeated dosage of skin homografts from the same donor to the same recipient. 3) By treatment of the recipient with various hormones. 4) Administration of X radiation to the skin graft after removal and just before grafting to the recipient.

Refrigeration of homografts.[1]—Thirty-seven homografts were stored for periods of time extending from 8 to 308 days. Groups of these homografts were kept at the following temperatures: 4° C., 0° C., −20° C., and −78° C. Loss of epithelium of these grafts, which was taken as the clinical sign of degeneration, became apparent as early as the fifth postoperative day and was delayed as long as the eighteenth day in one graft. Subsequent to loss of the epithelium, all the dermis sloughed at varying periods thereafter. Histologic studies of biopsies taken at intervals following grafting confirmed clinical observations and verified the conclusion that all homografts failed to survive permanently.

Repeated dosage of homografts.[2]—Three patients received more than one homograft of skin from the same donor—two, three, and five grafts, respectively. In the second patient the first-set homografts survived for 5 weeks, the second-set for 4 weeks, and the third-set for 3 weeks. The third patient received the most prolonged study of the series in that fresh homografts from the same donor were sutured to a freshly denuded raw surface, from which a contracted burn scar had been excised. This was repeated at weekly intervals. The first-set homograft survived for 12 days and subsequent grafts were lost with increasing rapidity, so that the fifth-set was lost on the fifth day. It was interesting that in spite of repeated administration of homologous skin from the same donor, the patient showed no obvious clinical signs of sensitization.

Treatment of recipient with various hormones.—Four homografts of skin were applied to three patients receiving approximately 100 mg. of corticotrophin daily. Therapy with the hormone was delayed until 15 days after the application of one homograft, started on the same day as skin grafting on two occasions, and in the fourth graft pretreatment with hormone was started 6 days before the homograft of skin was applied. In this way the timing of the effect of ACTH on the homografts was varied, so that possible differences in reaction during the stages of healing of the grafts could be observed.[3] In one instance the major blood groups and Rh factor of the donor and recipient of the graft were completely compatible, but the homograft sloughed in the customary fashion. In all patients, autografts of skin were applied at the same time. These took successfully, and a sequence of biopsies at weekly intervals, commencing on the seventh postoperative day, revealed that inflammatory and degenerative changes became evident in the homograft as early as 7 days postoperatively, while the autografts progressed to normal healing.[4]

To study the effect of cortisone,[5] another patient was pretreated with 200 mg. of this hormone daily for 7 days and postoperatively for 24 days. A thin homograft (0.008 inches thick) was sutured on a large granulating surface, and an autograft from the patient was applied to the granulating area as a control, on the same day. The homograft began to lose its epithelium on the eleventh day, and had practically disappeared by the seventeenth day postoperatively.

Because of the great dilution of orally or intramuscularly injected hormones, it was thought worth while to study the effect of Compound F injected beneath the freshly applied skin graft. Two patients were treated with this hormone. In one, after the homograft had been sutured to its bed, Compound F was injected copiously beneath the graft and the usual pressure dressing was applied. The epithelium began to separate two weeks later and the dermis sloughed shortly afterward. In the second patient this hormone was again injected copiously beneath the graft at the time

[1] BAXTER, H., and ENTIN, M. A.: Clinical study of the fate of homografts in man. Effect of repeated dosage from same donor and of refrigeration on these grafts. Am. J. Surg. 81: 285-294, 1951.

[2] BAXTER, H., SCHILLER, C., and WHITESIDE, J. H.: The influence of ACTH on wound healing in man. Plast. & Reconst. Surg. 7: 85-99, 1951.

[4] BAXTER, H., SCHILLER, C., WHITESIDE, J. H., LIPSHUTZ, H., and STRAITH, R. E.: The effect of ACTH on the survival of homografts in man. Plast. & Reconst. Surg. 7: 492-504, 1951.

[5] BAXTER, H., SCHILLER, C., WHITESIDE, J. H., and RANDALL, R. E.: Use of ACTH and cortisone in surgery. Am. J. Surg. 83: 374-378, 1952.

of operation and a pressure dressing was applied. Although there was some delay in healing of the graft, 14 days later it appeared quite healthy and it was considered safe to continue subcutaneous injections of hormone. Accordingly, 1 cc. of this hormone was injected as evenly as possible beneath an area of the homograft approximately two inches square, the remaining part of the homograft receiving only the application of hormone at the time of skin grafting. Then 1 cc. of hormone was injected beneath this part of the graft at weekly intervals after the fourteenth day until the entire graft was sloughing rapidly. Therefore, it appeared that very high concentrations of the hormone, which is much less soluble than cortisone and presumably would remain *in situ* for a number of days, did not materially prolong the life of the treated part of the homograft.

X radiation of skin graft.—In three patients, after homografts had been removed from the donor they were wrapped in sterile covering and one-half the graft was treated with X radiation, while the other half was completely sealed by lead plates. One patient received two homografts exposed to 1,500 r and 3,000 r, respectively. The second patient's homograft was given 3,000 r, while the third patient's graft was given 4,500 r. The irradiated portion of these homografts took almost as well as the control or unexposed portion. The subsequent course of these grafts was recorded by means of serial biopsies and color photographs that confirmed the clinical impression that treatment with X radiation in no way prolonged survival of the treated part of the homograft.

Summary.—It is quite apparent that these four methods of treating skin grafts in man did not prolong their survival, and offer no solution to the problem of obtaining permanent survival of skin homografts in man.

Dr. Bassett

At the Tissue Bank we have stored homogenous skin in a nutrient medium consisting of 10 percent pooled human serum and 90 percent balanced salt solution (Earle's) maintained at a temperature of 3° C. When so stored, skin may be demonstrated to maintain viability for periods in excess of 3 months. In a total of approximately 70 grafts of this material there was no indication that the survival period was increased over the usual 3- to 5-week period.

Dr. Talmadge Buchanan, University of Virginia, Charlottesville, Va.

In reply to Dr. Medawar's question as to whether or not the survival of autogenous skin transplants prepared and utilized by the freeze-drying technique persisted longer than homografts similarly prepared, the answer is No. In my experimental and clinical use of these transplants, it has been shown that the survival time or period of persistence is the same. The grafts do not become stromatized or vascularized.

Dr. J. Walter Wilson, Brown University, Providence, R. I.

One important cytological feature of Dr. Hauschka's observations has not been emphasized, that is that cells with abnormal chromosome complements continue to divide and thrive. It has been a biological dogma, based on Boveri's polyspermy experiments, that a somatic cell must have at least one complete set of chromosomes in order to maintain itself. It seems very unlikely that cells with reduced numbers of chromosomes derived from abnormal mitotic figures should receive a normal set of chromosomes. We have seen similar phenomena in mouse liver. I therefore think that it is possible that differentiated somatic cells can maintain and reproduce themselves without a normal set of chromosomes.

Conditioning of the Host [1]

HELENE WALLACE TOOLAN, PH.D., *Sloan-Kettering Institute, New York, N. Y.*

One of the methods used in investigating the dynamics underlying transplantation of tissue from one animal to another, has been the conditioning of the graft recipient so that its aggressive reaction toward a foreign tissue implant is reduced; a subsequent enhanced or permanent survival of the graft ensues. Considerable literature on the subject has now accumulated but, unfortunately, it is often confusing, for the means employed to alter the host resistance have been varied, the rationale on occasion obscure, and the results often difficult to interpret. Further, not all of the procedures are strictly comparable, for some appear to have achieved their effect merely by supplying a relatively better nutritive substrate for the implant while others have induced a true change, either temporary or permanent, in the host constitution. The present report is a review of the literature to date with emphasis on the principal methods of conditioning the host to a foreign tissue graft now employed by investigators in the field. An attempt will be made to relate the procedures where it seems possible.

Diet and Hyperemia

Since diet and hyperemia produce comparatively little genuine alteration in the inherent resistance of a host to a foreign implant, this subject will be treated first and briefly. It has long been accepted as an axiom that weak and undernourished animals are poor hosts for either spontaneous or transplanted tumor growth (*1–4*). As a corollary, some early investigators noted that animals fed on special diets were more susceptible to transplanted neoplasms. Van Alstyne and Beebe (*5*), for example, reported that a sarcoma of the Buffalo rat grew better when the hosts had been fed and were kept on a high-lactose carbohydrate diet, a finding which Woglom (*6*) was unable to confirm. More recently work on diet and tumor growth has concerned itself mainly with the need for adequate caloric intake and protein levels for tumor growth, the stimulatory effect of a high-fat-content diet on the incidence of spontaneous tumors and on the induction of neoplasms by carcinogenic agents (*7–10*), and the relation

[1] Presented at the Tissue Transplantation Conference, Arden House, Harriman, N. Y. ,October 8, 1952.

Page 746 (December 1953 issue): lines 13 to 16 from bottom of page state, "Baetzner and Beck (*20*) in a short note stated that prior treatment of a recipient with repeated injections of serum and tissue extracts from the donor enhanced subsequent growth of skin, 'inner organ,' and cartilage grafts." In Baetzner and Beck's paper the treatment was given *after* the transplantation rather than *prior* to it. *Inner organ* should have been translated *endocrine glands*. (In the paper "Conditioning of the Host," by Toolan.)

of certain vitamins, especially those of the B complex, to tumor incidence (*11, 12*). It has been learned that animals on a diet deficient in choline are very susceptible to neoplasms of the liver (*13, 14*), while numerous papers confirm the finding that riboflavin exerts an inhibitory influence on the formation of neoplasms induced by choline deficiency (*15*) and of hepatomas induced by feeding of the azo dyes (*16*). Literature on the relationship of these factors to transplanted tumors is limited.

Hyperemia of the implantation site has also been considered as stimulatory to tumor growth and, conversely, anemia as inhibitory (*17, 18*). Related to this observation may be the finding of Wallace *et al.* (*19*) that subcutaneous methylcholanthrene-induced tumors as well as a transplantable sarcoma of strain C3H mice, implanted subcutaneously, grew better and more rapidly in C3H mice kept in a room at a constant temperature of 92° F. than in litter mates kept at 65°. The authors stated that the constant vasodilatation of the blood vessels in the warm room might have provided an exceptionally optimal graft bed and thus accounted for their results.

It is again stressed that both adequate nourishment and an increased blood supply at the implantation site merely offer the most favorable conditions that can exist in a given resistant host. Growth enhancement of a graft in such a situation is not comparable to stimulatory effects obtained in the experiments now to be described, wherein the inherent resistance of the graft recipient has been affected.

Desensitization

It would seem that one of the most obvious approaches to the problem of promoting or prolonging graft growth would be an attempt to desensitize the host, yet comparatively few investigators have employed this method as such, though some of the results obtained in the work now being done with growth factors may be desensitization phenomena. Baetzner and Beck (*20*) in a short note stated that prior treatment of a recipient with repeated injections of serum and tissue extracts from the donor enhanced subsequent growth of skin, "inner organ," and cartilage grafts. It was the authors' theory that antibodies are produced against the foreign material which are more easily attached to the serum and extracts than to the grafts; the latter are then less inhibited than similar grafts placed in control hosts. Rohde (*21*), in contrast, found that treatment with blood plasma or serum, skin extracts or autolysates, increased rather than lowered resistance to skin homografts. In a series of unpublished experiments in our own laboratory our findings agreed with those of Rohde, for repeated injections of fresh tissue suspensions from organs and skin of the inbred strain of C3H mice rendered inbred strain A hosts completely resistant to a subsequently implanted C3H mammary carcinoma. In the control strain A mice, the C3H tumor took uniformly and grew progressively for 10 to 12 days before regression. Similar injections of cell suspensions from C3H mice into heterologous rat hosts pro-

duced results analogous to those obtained in the treated strain A mice. Usually, in untreated rats, a few identifiable mouse cells may be found for 4 to 7 days after implantation, a finding true for the control rats of these experiments. In the treated hosts, however, no mouse cells could be found on the fourth day. One of our observations was that even X-irradiated rats and strain A mice became completely resistant to the C3H carcinoma when they had been treated with C3H cell suspensions concomitantly with the X irradiation, prior to implantation of tumor. The possible significance of this finding will be discussed later in the section on irradiation. It is noteworthy that when the injections of cell suspensions were given *after* the implantation of the tumor, regression of the latter was delayed slightly in the homologous mouse hosts; there was also some indication that growth was prolonged in the X-irradiated rats.

Very recently, a report was published by Allen and co-workers (*22*) on the effect of desensitization of donor skin on the survival of skin homografts in rabbits. These investigators found that rabbits, injected with antigenic material prepared from the skin of a rabbit, subsequently supported skin homografts three times as long as control animals. The addition of ACTH to the antigen or the use of ACTH alone had no effect. After the grafts finally sloughed, intravenous injections of a specific skin antigen into the host animals caused anaphylactic shock and death—an observation supporting the role of an immune reaction in the failure of homograft survival.

The experiments just described which attempt to desensitize the graft host are probably closely allied to the later work done by investigators who are interested in "growth factors," a subject that will be discussed next. As the latter workers have pointed out, the host-tissue relationship and the dosage of material given are highly important in the interpretation of experimental results. Such considerations may account for the conflicting results obtained in the desensitization experiments.

Growth Factors

One of the most interesting methods of enhancing the growth of tissues in a foreign host has been the conditioning of recipient animals, prior to grafting, with a so-called "growth factor." The nature of these stimulating substances, even the question of whether they are the same or separate entities, is as yet undetermined though the first report of such a factor occurred as far back as 1907. At that time, when investigators were first working on the possibility of producing an immunity to transplanted tumors by the injection of nonliving material, Flexner and Jobling (*23*) observed that an emulsion of the Flexner-Jobling sarcoma of the rat, previously heated for an hour and a half at 56° C. and then injected into rats, caused these animals to be hypersusceptible to a graft of sarcoma implanted 10 to 30 days later. The tumors grew more rapidly and larger in treated animals than in control rats. No such effect was obtained with an unheated tumor emulsion, with bouillon, blood serum, or Ringer's solution. If the injections were repeated one or two times at intervals

of 10 days, the growth and persistence of the tumors was still further increased. Injection of the heated emulsion 24 hours after the implantation of the sarcoma did not produce the enhancement. In a second paper by these authors (*24*), they reported the results of testing possible growth-stimulating potentialities of sera from normal and immune rats (animals in which the tumor had regressed) and of heated and unheated emulsions of liver, spleen, kidney, muscle, and testicle. None were effective; instead they appeared to increase the host resistance. Studies were also made on immune hosts. When immune rats were treated with the heated tumor emulsion and 10 days later implanted with the sarcoma, it was found that tumors developed in 30 percent of the animals. They grew in only 9 percent of untreated immune hosts but in 100 percent of non-immune control rats. An interesting experiment is reported on a series of immune rats that had been injected with heated emulsion prior to their first tumor implantation. After these tumors had regressed, the now presumably immune animals were divided into two groups: one received a second injection of the heated emulsion, the other did not. All of them were implanted a second time with the sarcoma. The tumor grew in 30 percent of *both* groups. This was the same percentage as in the group of immune rats that were treated for the *first* time prior to their second implantation. It would seem from these results, that 30 percent of all the rats treated were affected in such fashion that they were *permanently* susceptible to repeated implantations of the tumor. In 1910, Jobling (*25*), using a carcinoma of the rat, repeated and confirmed his previous work on the sarcoma. He noted again that emulsions of heated and unheated liver, unheated spleen and tumor, induced resistance rather than susceptibility in the hosts. In this same year, Bashford and Russell (*26*) reported that a mouse tumor (#199), "disintegrated" by pounding in a mortar with ice, did not produce growths when injected into the usual mouse hosts but instead induced a hypersensitivity to a later graft of this tumor. Haaland (*27*), not distinguishing between the use of normal and malignant tissue, also reported that injections of killed cells made treated animals susceptible to the Bashford carcinoma 63. It was in this paper that his frequently quoted statement was made to the effect that only living cells could induce resistance to cancer. Leitch (*28*), in the same year, used a strain of mice in which the Bashford carcinoma regularly grew in only 33 percent of the animals and found that prior treatment of the mice with an emulsion of cells of Bashford tumor, previously frozen and apparently nonviable, raised the number of successful implantations to 78 percent.

After this early series of papers very little appeared on the subject of a growth-promoting factor for tumor transplants until 1924 when Chambers and Scott (*29*, *30*) reported that they had autolyzed the Jensen sarcoma of the rat at 42° C. in a toluene-saturated solution and obtained a clear fluid with only a trace of protein; this fluid, if inoculated into rats, made them hypersusceptible to the Jensen sarcoma of the rat. The authors felt that the vital substance was a nonliving, fairly stable, chemical body,

possibly related to the growth-promoting substances in the embryo extracts used for tissue culture (*31, 32*).

In the early 1930's, the first of a series of papers appeared by Casey and co-workers (*33*) on an enhancing substance that he has called the "XYZ" factor. Material prepared from a saline suspension of Brown-Pearce rabbit tumor, which had been anaerobically refrigerated for 2 weeks or longer, was injected into rabbits that were implanted 2 weeks later with fresh Brown-Pearce tumor. One hundred percent of the treated animals developed tumors, whereas 68 percent of the controls showed growth. The rate of tumor growth was also enhanced and the incidence and number of metastases as well (*34*). The factor was species-specific (*35-37*) for it did not enhance the Bashford carcinoma in mice nor did the material prepared in similar manner from the Bashford tumor affect the Brown-Pearce carcinoma or even the homologous Sarcoma 180, a mouse sarcoma, though it was effective for the tumor of origin. Rabbits, treated with the XYZ factor, remained hypersusceptible for as long as 7 months (*38*). About this time (1934) Spadafina (*39*) noted the presence of an enhancing factor for the Ehrlich carcinoma in a filtrate of this tumor, and shortly thereafter Casey (*40*) reported that a cell-free filtrate of the Brown Pearce carcinoma was as effective as the unfiltered, heated tissue emulsions in promoting growth of this tumor. Further work on the XYZ factor has indicated that it withstands drying and is thermolabile (56° C.) (*41*), differs from the spreading factor of Duran-Reynals (*42*), is not present in filtrates of the testicle or spleen of normal rabbits or rabbits injected with the factor (*43*), and cannot be recovered from filtrates of fresh tissue of Brown-Pearce tumor (*44*). It has been suggested by Casey and co-workers that the XYZ factor must be bound in fresh tissue but activated by freezing. Two recent papers by this group of investigators, on the XYZ factor in the E0771 carcinoma of the mouse, have pointed out that the growth substance is equally effective in various strains of mice as long as the tumor cells are viable in the hosts tested (*45*); and they have also made the most interesting observation that E0771 carcinomas from animals treated with the XYZ factor maintained their enhanced growth rate for at least six passages in untreated hosts (*46*). The authors suggest that this increase in growth rate is due either to a mutation in the tumor or to a variation induced by the XYZ treatment.

A group of workers at the Jackson Memorial Laboratory have reported (*47*) that transplantable tumors of the mouse could either be stimulated or inhibited by multiple prior injections of *lyophilized* tumor tissue, depending on the relationship of the tumor and stocks employed. When mice of strains C57BR, C57BL, or BALB/c were treated with 21 intraperitoneal injections of lyophilized strain A carcinoma 15091a, a suspension of fresh tumor cells subsequently implanted grew progressively until death of the hosts, though in control animals only small nodules appeared which soon regressed. However, when C57L mice were given 21 intraperitoneal injections of lyophilized strain C57BL

myeloid leukemia C1498, injections of fresh tumor failed to grow in 54 percent of the animals; none of the controls were negative. The authors explain that the diverse results obtained are due to the complexity of the genetic basis for tissue specificity. They suggest, in a second paper (*48*), that there are at least four variables which may produce numerous combinations: *a*) the tumor used as lyophilized tissue; *b*) the tumor used as fresh tissue; *c*) the host strain; and *d*) the dosage of lyophilized tissue. Using nine tumors and seven different strains of mice, they obtained results varying from almost complete inhibition to strong stimulation, depending on the host-tissue combination. They also report that the dosage is highly important.

Kaliss and co-workers (*49*, *50*) have also used lyophilized tissues and with this type of material found a substance in normal tissues of the mouse, as well as in tumor, that would enhance the growth of a tumor homotransplant in strains of mice in which it would ordinarily regress. The tumor used was the spindle-cell carcinoma 15091a from strain A mice and the hosts were C57BL and C57BR mice. The enhancing substance was not present in an ultrafiltrate of fresh tumor (*51*). These investigators feel that their enhancing material is similar to the XYZ factor of Casey and state that the reason Casey was unable to find this factor in testis and spleen extracts of normal rabbits was probably due to a genetic difference between the tumor and these tissues. Kaliss and Newton (*52*) also noted that the quantity of lyophilized tissue used for conditioning the host was of prime importance since small doses caused inhibition of tumor, large doses an enhancement, and intermediate amounts apparently no effect. An interesting finding of Kaliss and Molomut (*53*) was that tissue antisera, prepared by the injection of spleen, kidney, and tumor 15091a of strain A mice into rabbits, also appeared to possess an enhancing factor, for such sera injected into C57BL mice produced a marked stimulation in the growth of two strain A tumors in those mice. Recently Kaliss and Spain (*54*) reported that prior injections of lyophilized tissues of the mouse appeared to have some effect on the survival of normal tissue grafts as well as on tumor, since spleen survived for 3 months in 25 to 40 percent of treated male mice while none remained alive in the controls. This is an observation of possibly considerable significance to clinicians working with grafts of human skin. In a summary of his observations to date, Kaliss (*55*) postulated that the effect of any of the so-called "growth" factors is on the inherent resistance of the host, either leading to its increase or decrease. Thus, it is not correct to speak of "stimulation" of a tumor for, in view of the experimental results obtained, two factors would then have to be involved, one stimulatory, the other inhibitory.

Finally, discussion of the growth factors would not be complete without mention of the work of Cloudman (*56*) and Law (*57*) who found that resistance to certain transplanted tumors was reduced by foster nursing of the refractory mice by mothers of a susceptible strain.

In summary, the question of just what the growth factor or factors are, remains unanswered. Whether it is an enzyme or catalyst as suggested

by Chambers and Scott (*30*), a virus, a submicroscopic particle similar to the one reported by Kidd (*58*, *59*) as a constituent of the Brown-Pearce tumor cell, or whether it is an expression of individuality differentials applying to both normal and malignant tissues as held by Kaliss (*50*) and Snell (*60*) remains to be discovered.

Vital Staining

One of the well known but comparatively little used methods for lowering host resistance to tumor grafts has been the employment of vital dyes. Ludford (*61*, *62*) injected mice with both trypan blue and vital red, and also with inorganic colloids prior to an implantation of mouse carcinoma 63, and reported that both natural resistance and that induced by embryo skin were lowered. It was his theory that the dyes and colloids overloaded the reticuloendothelial system which he believed was associated with the host's aggressive reaction toward an implant. Foulds (*63*) gave rabbits 4 to 6 injections of trypan blue before an intravenous injection of Brown-Pearce tumor cells and found that the incidence of tumors in the spleen, lung, and liver was increased. Saphir and Appel (*64*) also worked with rabbits and noted that "takes" from an intravenous injection of Brown-Pearce tumor cells occurred in 7 of 9 immune animals previously treated with prolonged intravenous administration of trypan blue. Andervont (*65*, *66*) observed that prior injections of trypan blue destroyed an acquired resistance to Sarcoma 180 of the mouse and that the resistance of vitally stained strain D mice was lowered to such an extent that strain A or strain C3H tumors that ordinarily regressed grew progressively and went through several passages in them. He also found that tumors induced by a carcinogen appeared earlier in treated animals than in controls. Recently, Cohen and Cohen (*67*) have reported that when rats in which a transplanted hepatoma had a high percent of regression were either treated with trypan blue or X irradiated, their regressing tumors again began to grow, killing the animals. The authors stress that the effects of trypan blue and X irradiation are analogous. However, it is not certain that this conclusion is justified, for both Saphir and Appel, and Andervont, as well as Ludford, have indicated that trypan blue breaks down resistance in the immune host, while investigators working with irradiated animals do not always report this finding.

X Irradiation

The pioneer work of Murphy (*68*) on X irradiation of hosts, in order to make them hypersusceptible to implantations of homologous and heterologous tissue, is so well known that it will be summarized here rather briefly. Murphy contended that the lymphocyte was the embodiment of resistance to foreign tissue and that any procedure that would reduce these cells would enhance inversely the survival of transplants. The principal procedure he employed to eliminate the lymphoid tissue was X irradiation and, using this means, he reported that positive results in homologous

transplantation were increased, *i.e.* that tumors that ordinarily would regress grew progressively until death of the host (*69*), that immunity to homologeous neoplasms could be overcome (*70*), and that heterologous transplants of the Ehrlich carcinoma of the mouse could be effected in rats even though the tumors eventually regressed (*71*). At no time did Murphy consider that the lymphoid cell might be just one factor in an over-all immune response. He looked upon these cells as an immediate defense against the foreign material, as is shown by his papers on local irradiation (*72*) and heat application (*73*) as a method of increasing or decreasing the cellular response in limited areas that were then resistant or susceptible to the grafts.

Other workers since Murphy have reported that resistance to homologous tumor grafting is broken down by prior irradiation of the host (*74–77*). Mottram (*78*) has noted also that rats immune to the Jensen sarcoma of the rat could be rendered susceptible by such treatment; his results are thus similar to those obtained by the investigators using trypan blue. Clemmesen (*79*) has supported the finding that tumors could be successfully transferred to X-irradiated heterologous hosts by his serial passages of Sarcoma 180 in X-irradiated rats. He pointed out, however, that acquired immunity toward heterologous grafts could not easily be broken down by irradiation, for he was unable to implant Sarcoma 180 successfully in rats in which this tumor had previously grown and regressed even though the hosts were irradiated prior to the second implantation (*80*). He considered that the effect of X irradiation on heterologous grafting was to delay the immune response, which, once present, was radiation-resistant. This was the same conclusion reached by Dempster *et al.* (*81*) who worked with noninbred homologous hosts and found that the survival of skin homotransplants in rabbits was prolonged by prior X irradiation of the hosts, but that survival of a second set of grafts from the same donor was not affected by a second prior irradiation. The authors suggested that there might be two factors involved in the host reaction: the first, a highly radiosensitive receptor, which initiates the immune response; and the second, the effector, which manufactures antibody and is radioresistant. In relation to these findings, the work of Bichel and Holm-Jensen (*82, 83*) on parabiosis and resistance to transplantation may be noted. These investigators reported that two tumors of the mouse that grew temporarily in X-irradiated rats could not be implanted successfully in X-irradiated animals joined parabiotically with nonirradiated hosts. The authors concluded that humoral factors must be responsible for determining resistance.

In our own work (*84*), we have found that a large percentage of human tumors, especially epidermoid carcinomas (received fresh and uncontaminated from the operating room), can be grown successfully and transferred serially in X-irradiated rats, mice and hamsters. Weanling rats, 25 to 35 days of age, have proved the most suitable of the hosts tested, for implantations of minced human tumor suspensions placed in these young hosts become well vascularized and grow rapidly for 14 to

20 days before regression occurs (figs. 1 to 9). This is a period long enough to be of practical value in testing various oncolytic agents (*85*). It is of interest to note that X-irradiated, newborn, nursing rats which are highly resistant to irradiation effects, have proved to be poor hosts for the human tumors. In X-irradiated weanling animals, the neoplasms actually seem to grow better than in the original human hosts, possibly because necrotic cells present in the graft are removed rapidly by the rat phagocytes and only the most vigorous and "healthy" cells remain. This observation, that growth of the human tumors in irradiated animals is unusually active, is borne out by the comparative ease with which the tumors of humans grown in the rat are propagated in tissue culture and on the chick membrane (figs. 10, 11), while the original tumor either fails to proliferate or grows poorly. The human neoplasms have retained, at all times, their particular morphology. We also have been able to grow, and transplant serially, nonmalignant human tissue such as skin (*86*) and colloid goiter in X-irradiated animals, an indication to us that the determining factor for successful heterologous transplantation is not the autonomy of the tissue used as a graft, but rather the diminution of resistance of the heterologous host—in this instance, by previous irradiation. It should be noted that the irradiated animals in which six different thyroid goiters were grown, had intact thyroids of their own.

In agreement with Clemmesen and Dempster, we have found that rats in which the human tumors have regressed are not made susceptible by a second irradiation to another graft even though the lymphoid count is drastically reduced. We also have been unable to prolong the survival of transplants by small doses of radiation during the period the graft is being supported. Finally, as has been previously noted, we have found that X-irradiated rats and even strain A mice are resistant to grafts of a carcinoma of strain C3H mice if, during the irradiation period, the hosts have been treated with repeated injections of cell suspensions from normal C3H organs and have thus become immune to C3H tissue. We have not as yet tested the effect of treatment with trypan blue on the resistance of such hosts.

ACTH and Cortisone

Recently the adrenal hormone, cortisone, and its related pituitary hormone, ACTH, have been employed to modify host resistance. Unlike the procedures that have been reviewed, use of these two substances has appealed more to the clinician than to the laboratory investigator as a possible practical means of promoting the "takes" of homologous skin grafts. Since this is a subject which is being discussed elsewhere at these meetings by Dr. Longmire, it will be touched on but briefly for the purpose of continuity.

Early observations indicated that cortisone inhibited all the elements of granulation tissue (*87*), delayed wound healing (*88*), decreased circulating antibodies (*89*), and modified sensitivity reactions (*90*). When Whitelaw (*91*) reported that treatment with ACTH had caused survival

of skin homografts in a patient with severe burns, surgeons and laboratory workers alike were stimulated to test the effect of the two hormones on skin homografting. Conflicting reports have ensued.

As has already been noted, Allen and co-workers (*22*) were unable to prolong the survival of skin homografts in rabbits by use of ACTH, a finding corroborated by other investigators (*92–95*) using ACTH and cortisone on laboratory animals, and by clinicians (*96–98*) employing these two hormones on patients with skin grafts. Billingham *et al.* (*99*), however, have reported that daily injections of cortisone lengthen the life of skin homografts in rabbits by a factor of three to four, and they have pointed out that similar beneficial effects can be obtained by entirely local application of the hormone (*100*). Maumenee (*101*) has observed that administration of cortisone can prevent clouding of corneal grafts in rabbits. It is his opinion that clouding in corneal transplants is an allergic response prevented by the cortisone.

Comparatively little work has been done on the use of either ACTH or cortisone to enhance tumor transplantation in animals, possibly because early reports indicated that these substances inhibited growth of established tumors (*102*, *103*). Three papers (*104–106*) have appeared which report that transplantable tumors of mice that would ordinarily regress or do poorly have grown progressively in the homologous hosts treated with cortisone. In one of these publications, Foley (*106*) noted that the progressive growth under the influence of cortisone treatment was limited to certain tumors in certain strains of mice, again a matter of host-tissue relationship. He also reported that when CF_1 mice were immune to the lymphosarcoma 6C3HED after growth and regression of this tumor in them, the immunity could not be broken down by cortisone treatment.

An interesting finding is that of Agosin *et al.* (*107*) who noted that cortisone arrested the local growth of a transplantable mammary carcinoma in strain C3H mice thus allowing a long period of survival during which metastases, which never occurred in untreated animals, became numerous. The interpretation of this finding is left open. It would seem that the metastases probably occurred as a result of the lengthened life of the treated animals, while the inhibition may have been related to an alteration in the stromal substrate unfavorable to local tumor spread—possibly a necessary factor for continued "healthy" growth.

At the present time we are in the process of testing the effects of cortisone on the growth of human tumors in heterologous hosts. Our experiments are unfinished and the interpretations thereof vastly complicated by the many known side effects of this hormone. We feel that such factors as the delay in inflammatory response (*108*, *109*), the relative impermeability of blood vessels (*110*, *111*) the inhibition of hyaluronidase (*112*), and alteration of the connective-tissue substrate possibly by removal of SH groups (*113*, *114*) have such considerable effects on graft survival from its earliest "take" through the period of established growth that it is difficult to evaluate just what the cortisone may be doing to counteract either the natural or immune resistance. It may be said, how-

ever, that X irradiation and cortisone appear to be synergistic, a finding which Syverton *et al.* (*115*) have reported in the production of lethal infections, for the human tumors are growing more rapidly and with a higher percentage of "takes" in X-irradiated animals treated with cortisone than in those X irradiated alone. There is also indication that tumors grow better in nonirradiated animals treated with cortisone than in the hosts X irradiated alone. At this date most of the enhancing effect of the cortisone seems to be due to an alteration of the initial natural resistance of the rat hosts, possibly, as mentioned, by its profound effect on the substrate provided, rather than on an acquired immunity.

General Considerations

It has not been possible to review in a limited time all the many factors associated with stimulation of graft growth. The large literature on the relation of endocrine factors, other than cortisone and ACTH, to tumor growth which has been reviewed extensively by Gardner (*116*) and Selye (*117*) will not be discussed here. The related problem of Halsted's law (*118, 119*) that an endocrine organ is more likely to be successfully transplanted when a deficiency of its particular secretion exists, bears note, in view of our finding that human thyroid not only survived in X-irradiated rats but also proliferated and appeared histologically active, even though the host thyroids were intact. Papers by Turner (*120, 121*) and Woodruff and Woodruff (*122*) have indicated that it is not directly the deficiency of the endocrine tissue which insures survival of the implant but rather the secretion by the pituitary of trophic hormone which in turn stimulates the graft. Further studies will have to be done with transplantations of endocrine organs into X-irradiated rats before any generalizations can be made on the pertinency of our findings to Halsted's dictum.

Summary

In summary, the experiments herein reviewed indicate that host resistance to foreign tissue implants can be reduced by various means, the rationale for which is not always clear though the over-all pattern presented is one of interference with an immune response of the host. X irradiation and cortisone may reduce both the natural resistance by alteration of the host connective tissue substrate and the acquired immunity by delay of antibody response. The total effect is probably a sum of diverse actions on the treated animal rather than one simple effect. Trypan blue and inorganic colloids appear to act by interfering with the acquired immunity, possibly by overloading cells responsible for, or related to, antibody production. They have not been tested on heterologous hosts so their effect on natural resistance is unknown. Finally, it would seem that the "growth factors" represent antigenic substances which cause an expression of the genetic elements comprising tissue specificity and therefore enhance or reduce resistance on the basis of host-tissue relationships.

References

(*1*) Rous, P.: The influence of diet on the transplanted and spontaneous mouse tumors. J. Exper. Med. 20: 433–451, 1914.

(*2*) Sugiura, K., and Benedict, S. R.: Influence of insufficient diets upon tumor recurrence and growth in rats and mice. J. Cancer Res. 10: 309–318, 1926.

(*3*) Tannenbaum, A.: On some aspects of diet and cancer. A. A. A. S. Cancer Res. Conference, Washington, D. C., 288–290, 1945.

(*4*) ———: Effects of varying caloric intake upon tumor incidence and tumor growth. Ann. N. Y. Acad. Sc. 49: 5–18, 1947.

(*5*) Van Alstyne, E. V., and Beebe, S. P.: Diet studies in transplantable tumors. J. Med. Res. 29: 217–232, 1913–1914.

(*6*) Woglom, W.: Diet and tumor growth. J. Exper. Med. 22: 766–779, 1915.

(*7*) Watson, A. F., and Mellanby, E.: Tar cancer in mice. II. The condition of the skin when modified by external treatment or diet, as a factor in influencing the cancerous reaction. Brit. J. Exper. Path. 11: 311–322, 1930.

(*8*) Bauman, C. A., Jacobi, H. P., and Rusch, H. P.: The effect of diet on experimental tumor production. Am. J. Hyg. 30: 1–6, 1939.

(*9*) Tannenbaum, A.: The genesis and growth of tumors. III. Effects of a high-fat diet. Cancer Research 2: 468–475, 1942.

(*10*) Dunning, W. F., Curtis, M. R., and Maun, M. E.: The effect of dietary fat and carbohydrate on diethylstilbestrol-induced mammary cancer in rats. Cancer Research 9: 354–361, 1949.

(*11*) Williams, R.: B vitamins and cancer. A. A. A. S. Cancer Res. Conference, Washington, D. C., 253–266, 1945.

(*12*) Morris, H. P.: Effects on the genesis and growth of tumors associated with vitamin intake. Ann. N. Y. Acad. Sc. 49: 119–139, 1947.

(*13*) Copeland, D. H., and Salmon, W. D.: The occurrence of neoplasms in the liver, lungs and other tissues of rats as a result of prolonged choline deficiency. Am. J. Path. 22: 1059–1079, 1946.

(*14*) Engel, R. W., Copeland, D. H., and Salmon, W. D.: Carcinogenic effects associated with diets deficient in choline and related nutrients. Ann. N. Y. Acad. Sc. 49: 49–67, 1947.

(*15*) Schaefer, A. E., Copeland, D. H., Salmon, W. D., and Hale, O. M.: The influence of riboflavin, pyridoxine, inositol, and protein depletion-repletion upon the induction of neoplasms by choline deficiency. Cancer Research 10: 786–792, 1950.

(*16*) Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P.: Partial protection of rats by riboflavin with casein against liver cancer caused by dimethylaminoazobenzene. Science 93: 308–310, 1941.

(*17*) Sittenfield, M. J.: Influence of anemia and hyperemia on the growth of sarcoma in the white rat. Proc. Soc. Exper. Biol. & Med. 9: 56–57, 1911.

(*18*) Algire, G. H., and Legallais, F. Y.: Vascular reactions of normal and malignant tissues *in vivo*. IV. The effect of peripheral hypotension on transplanted tumors. J. Nat. Cancer Inst. 12: 399–421, 1951.

(*19*) Wallace, E. W., Wallace, H. M., and Mills, C. A.: Effects of climatic environment upon the genesis of subcutaneous tumors induced by methycholanthrene and upon the growth of a transplantable sarcoma in C3H mice. J. Nat. Cancer Inst. 3: 99–110, 1942.

(*20*) Baetzner, W., and Beck, S.: Über Homoiotransplantation. Zentralbl. Chir. 55: 272, 1928.

(*21*) Rohde, C.: Über Versuche zur Überwindung der Anheilungschwerigkeiten homoplastischer Transplantate. Beitr. klin. Chir. 134: 11–127, 1925.

(*22*) Allen, H., Williams, R., Lovingood, C., and Ellison, E.: The effect of donor skin desensitization and ACTH on survival of skin homografts in rabbits. Ann. Surg. 135: 239–244, 1952.

(23) FLEXNER, S., and JOBLING, J. W.: On the promoting influence of heated tumor emulsions on tumor growth. Proc. Soc. Exper. Biol. & Med. 4: 156–157, 1907.
(24) ———: Restraint and promotion of tumor growth. Proc. Soc. Exper. Biol. & Med. 5: 16–18, 1907.
(25) JOBLING, J. W.: Biology of the mixed tumor of the rat. Monographs Rockefeller Inst. Med. Res. 1: 52–72, 1910.
(26) BASHFORD, E. F., and RUSSELL, B.: Further evidence on the homogeneity of the resistance to the implantation of malignant new growths. Lancet, London 1: 782–787, 1910.
(27) HAALAND, M.: The contrast in the reactions to the implantation of cancer after the inoculation of living and mechanically disintegrated cells. Lancet, London 1: 787–789, 1910.
(28) LEITCH, A.: Experimental diminution of resistance to mouse cancer. Lancet, London 1: 991–992, 1910.
(29) CHAMBERS, H., and SCOTT, G.: Experiments on immunity to tumour growth. Brit. J. Exper. Path. 5: 1–12, 1924.
(30) ———: On a growth-promoting factor in tumour tissue. Brit. J. Exper. Path. 7: 33–40, 1926.
(31) CARREL, A.: Artificial activation of the growth *in vitro* of connective tissue. J. Exper. Med. 17: 14–19, 1913.
(32) FISCHER, A.: The nature of the growth-promoting substances in the embryonic tissue juice. Acta. physiol. Scandinav. 3: 54–70, 1941.
(33) CASEY, A. E.: Experimental enhancement of malignancy in the Brown-Pearce rabbit tumor. Proc. Soc. Exper. Biol. & Med. 29: 816–818, 1932.
(34) ———: The experimental alteration of malignancy with an homologous mammalian tumor material. Am. J. Cancer 21: 760–775, 1934.
(35) ———: A species limitation of an enhancing material derived from a mammalian tumor. Proc. Soc. Exper. Biol. & Med. 30: 674–677, 1933.
(36) ———: Failure of a mouse carcinoma material to enhance a mouse sarcoma. Proc. Soc. Exper. Biol. & Med. 30: 1025–1026, 1933.
(37) ———: Specificity of enhancing materials from mammalian tumors. Proc. Soc. Exper. Biol. & Med. 31: 663–665, 1934.
(38) ———: A persistant hypersusceptibility induced in rabbits with an homologous tumor material. Proc. Soc. Exper. Biol. & Med. 31: 666–667, 1934.
(39) SPADAFINA, L.: La chute des résistances antinéoplastiques avec le filtrat tumoral. Cancer, Bruxelles 11: 1–24, 1934.
(40) CASEY, A. E.: Experimental alteration of malignancy with homologous mammalian tumor material. III. Concerning filterability of material. Am. J. Cancer 26: 276–290, 1936.
(41) ———: Experiments with a material from the Brown-Pearce tumor. Cancer Research 1: 134–135, 1941.
(42) ———: Comparison of the effect of an homologous tumor material and the Duran-Reynals factor on tumor growth. Am. J. Cancer 35: 354–362, 1939.
(43) DRYSDALE, G., and CASEY, A. E.: Search for the Brown-Pearce tumor XYZ factor in rabbit spleen. Proc. Soc. Exper. Biol. & Med. 69: 306–308, 1948.
(44) CASEY, A. E., DRYSDALE, G., ROSS, G. L., and ELROD, B. A.: Filtrates of fresh tumor injected prior to transplantation of the homologous tumor. Proc. Soc. Exper. Biol. & Med. 77: 221–224, 1951.
(45) CASEY, A. E., ROSS, G. L., and LANGSTON, R.: Selective XYZ factor in C57 black mammary carcinoma Eo771. Proc. Soc. Exper. Biol. & Med. 72: 83–89, 1949.
(46) CASEY, A. E., LASTER, W. R., JR., and ROSS, G. L.: Sustained enhanced growth of carcinoma E0771 in C57 black mice. Proc. Soc. Exper. Biol. & Med. 77: 358–362, 1951.
(47) SNELL, G. D., CLOUDMAN, A. M., FAILOR, E., and DOUGLASS, P.: Inhibition and stimulation of tumor homoiotransplants by prior injections of lyophilized tumor tissue. J. Nat. Cancer Inst. 6: 303–316, 1946.

(*48*) SNELL, G. D., CLOUDMAN, A. M., and WOODWORTH, E.: Tumor immunity in mice induced with lyophilized tissue, as influenced by tumor strain, host strain, source of tissue and dosage. Cancer Research 8: 429–437, 1948.

(*49*) KALISS, N., and SNELL, G. D.: The effect of prior injections of lyophilized normal mouse tissues on the subsequent growth of a transplanted tumor. (Abstract.) Anat. Rec. 105: 572–573, 1949.

(*50*) ———: The effects of injections of lyophilized normal and neoplastic mouse tissues on the growth of tumor homoiotransplants in mice. Cancer Research 11: 122–126, 1951.

(*51*) KALISS, N., JONAS, G., and AVNET, N.: Growth enhancement of tumor homoiotransplants in mice following injections of homogenates and ultrafiltration sediments of mouse tissues. (Abstract.) Cancer Research 10: 228, 1950.

(*52*) KALISS, N., and NEWTON, O.: Effect of injection dosage level of lyophilized mouse tissue on the subsequent growth of a tumor homoiotransplant. Anat. Rec. 105: 535–536, 1949.

(*53*) KALISS, N., and MOLOMUT, N.: The effect of prior injections of tissue antiserums on the survival of cancer homoiografts in mice. Cancer Research 12: 110–112, 1952.

(*54*) KALISS, N., and SPAIN, D.: The effect of prior injections of lyophilized mouse tissues on the survival of normal tissue homoiografts in mice. (Abstract.) Cancer Research 12: 272, 1952.

(*55*) KALISS, N.: Regression or survival of tumor homoiografts in mice pretreated with injections of lyophilized tissues. Cancer Research 12: 379–382, 1952.

(*56*) CLOUDMAN, A.: The effect of an extra-chromosomal influence upon transplanted spontaneous tumors in mice. Science 93: 380–381, 1941.

(*57*) LAW, L. W.: Foster nursing and the growth of transplantable leukemias in mice. Cancer Research 2: 108–115, 1942.

(*58*) KIDD, J. G.: A distinctive substance associated with the Brown-Pearce rabbit carcinoma. I. Presence and specificity of the substance as determined by serum reactions. J. Exper. Med. 71: 335–350, 1940.

(*59*) ———: A distinctive substance associated with the Brown-Pearce rabbit carcinoma. II. Properties of the substance: discussion. J. Exper. Med. 71: 351–371, 1940.

(*60*) SNELL, G. D.: The immunogenetics of tumor transplantation. Cancer Research 12: 543–546, 1952.

(*61*) LUDFORD, R. J.: Resistance to growth of transplantable tumours. I. Influence of vital staining on induced resistance. Brit. J. Exper. Path. 12: 45–49, 1931.

(*62*) ———: Resistance to the growth of transplantable tumours. II. Influence of dyestuffs and colloids on natural resistance. Brit. J. Exper. Path. 12: 108–113, 1931.

(*63*) FOULDS, L.: Effect of vital staining on distribution of the Brown-Pearce rabbit tumour. Scient. Rep. Invest. Imp. Cancer Res. Fund. 10: 21–31, 1932.

(*64*) SAPHIR, O., and APPEL, M.: Attempts to abrogate immunity to the Brown-Pearce carcinoma. Cancer Research 3: 767–769, 1943.

(*65*) ANDERVONT, H.: Studies on immunity induced by mouse Sarcoma 180. Pub. Health Rep. 47: 1859–1877, 1932.

(*66*) ———: The influence of trypan blue upon the resistance of mice to transplantable and induced tumors. Pub. Health Rep. 51: 591–600, 1936.

(*67*) COHEN, A., and COHEN, L.: Analogous effects of radiation and trypan blue on rats resistant to transplanted hepatoma. Nature, London 167: 1063, 1951.

(*68*) MURPHY, J. B.: The lymphocyte in resistance to tissue grafting, malignant diseases and tuberculous infection. Monographs Rockefeller Inst. Med. Res. 21: 1–168, 1926.

(*69*) MURPHY, J. B., and MORTON J.: The lymphocyte in natural and induced resistance to transplanted cancer. J. Exper. Med. 22: 204–211, 1915.

(*70*) MURPHY, J. B., and TAYLOR, H. D.: The lymphocyte in natural and induced resistance to transplanted cancer. III. The effect of X-rays on artificially induced immunity. J. Exper. Med. 28: 1–9, 1918.

(*71*) MURPHY, J. B.: Heteroplastic tissue grafting effect through roentgen-ray lymphoid destruction. J.A.M.A. 62: 1459, 1914.

(*72*) MURPHY, J. B., HUSSEY, R., NAKAHARA, W., and STURM, E.: Studies on X-ray effects. VI. Effect of the cellular reaction induced by X-rays on cancer grafts. J. Exper. Med. 33: 299–313, 1921.

(*73*) MURPHY, J. B., and STURM, E.: The lymphocyte in natural and induced resistance to transplanted cancer. IV. Effect of dry heat on resistance to transplanted cancer in mice. J. Exper. Med. 29: 25–30, 1919.

(*74*) CHAMBERS, H., SCOTT, G., and RUS, S.: On the action of X-rays upon the transplantation of a spontaneous carcinoma of the rat. J. Path. & Bact. 23: 384–387, 1920.

(*75*) WAGNER, A. (Aarhus): Investigations concerning the influence of roentgen irradiation on the resistance to cancer in white mice; preliminary communication. Acta radiol. 10: 539–553, 1929.

(*76*) KREBS, K., RASK-NIELSEN, H., and WAGNER, A.: Origin of lymphosarcomatosis and its relation to other forms of leucosis in white mice. Acta radiol. (Supplement) 10: 1–53, 1930.

(*77*) FURTH, J., SIEBOLD, H., and RATHBONE, R.: Experimental studies on lymphomatosis of mice. Am. J. Cancer 19: 521–604, 1933.

(*78*) MOTTRAM, J. C., and RUSS, S.: Observations and experiments on the susceptibility and immunity of rats towards Jensen's rat sarcoma. Proc. Roy. Soc., London, s.B. 90: 1–33, 1917–19.

(*79*) CLEMMESEN J.: On transplantation of tumor cells to normal and pre-irradiated heterologous organisms. Am. J. Cancer 29: 313–332, 1937.

(*80*) ———: Influence of roentgen radiation on heterologous and homologous transplantation immunity. Am. J. Cancer 38: 483–491, 1940.

(*81*) DEMPSTER, W., LENNOX, B., and BOAG, J. W.: Prolongation of survival of skin homotransplants in the rabbit by irradiation of the host. Brit. J. Exper. Path. 31: 670–679, 1950.

(*82*) BICHEL, J., and HOLM-JENSEN, I.: Parabiosis and resistance to transplantation. I. Influence of parabiosis on the growth of mouse leukosis in irradiated rats. Acta path. et microbiol. Scandinav. 24: 531–538, 1947.

(*83*) ———: Parabiosis and resistance to transplantation. II. The influence of parabiosis on the growth of a mouse carcinoma and a mouse sarcoma in irradiated rats. Acta path. et microbiol. Scandinav. 25: 540–542, 1948.

(*84*) TOOLAN, H. W.: Successful subcutaneous growth and transplantation of human tumors in X-irradiated laboratory animals. Proc. Soc. Exper. Biol. & Med. 77: 572–578, 1951.

(*85*) TOOLAN, H. W., and MOORE, A. E.: Oncolytic effect of Egypt virus on a human epidermoid carcinoma grown in X-irradiated rats. Proc. Soc. Exper. Biol. & Med. 79: 697–702, 1952.

(*86*) TOOLAN, H. W.: Proliferation and vascularization of adult human epithelium in subcutaneous tissues of X-irradiated heterologous hosts. Proc. Soc. Exper. Biol. & Med. 78: 540–543, 1951.

(*87*) RAGAN, C., HOWES, E. L., PLOTZ, C., MEYER, K., and BLUNT, J.: Effect of cortisone on production of granulation tissue in the rabbit. Proc. Soc. Exper. Biol. & Med. 72: 718–721, 1949.

(*88*) PLOTZ, C. M., BLUNT, J. W., and RAGAN, C.: Retardation of wound healing by cortisone. Surgery 28: 177–181, 1950.

(*89*) BJØRNEBOE, M., FISCHEL, E., and STOERK, H.: The effect of cortisone and adrenocorticotrophic hormones on the concentration of circulating antibody. J. Exper. Med. 93: 37–49, 1951.

(*90*) HARRIS, S., and HARRIS, T.: Effect of cortisone on some reactions of hypersensitivity in laboratory animals. Proc. Soc. Exper. Biol. & Med. 74: 186–189, 1950.

(*91*) WHITELAW, M.: Physiological reaction to pituitary adrenocorticotropic hormone (ACTH) in severe burns. J.A.M.A. 145: 85–88, 1951.

(*92*) Weisman, P., Quinby, W. C., Jr., Wight, A., and Cannon, B.: The adrenal cortical hormones and homografting; exploration of a concept. Ann. Surg. 134: 506–515, 1951.

(*93*) Martin, B., Morgan, T., and Lovingood, C.: The effect of ACTH on autogenous and homologous skin grafts in rabbits. Surgery 31: 258–262, 1952.

(*94*) Marrangoni, A.: The effects of cortisone on skin transplants in the experimental animal. *In* Surgical Forum (1951). Clin. Cong. Am. Coll. Surg. Philadelphia, W. B. Saunders Co., 1952, pp. 462–468.

(*95*) Randall, P., Brown, J., and McDowell, G.: The effects of ACTH and cortisone on experimental skin homografts. *In* Surgical Forum (1951). Clin. Cong. Am. Coll. Surg. Philadelphia, W. B. Saunders Co., 1952, pp. 475–481.

(*96*) Ellison, E., Martin, B., Williams, R., Clatworthy, H., Hamwi, G., and Zollinger, R.: The effect of ACTH and cortisone on the survival of homologous skin grafts. Ann. Surg. 134: 495–505, 1951.

(*97*) Trusler, H., Glanz, S., and Bauer, T.: An evaluation of pituitary adrenocorticotropic hormone (ACTH) in the treatment of severe burns: relationship to skin grafting. Plast. & Reconstruct. Surg. 9: 478–489, 1952.

(*98*) Butterfield, W., Williams, A., and Evans, E.: Effect of adrenocorticotropic hormone on the survival of homografts. Lancet, London 262: 737, 1952.

(*99*) Billingham, R., Krohn, P., and Medawar, P.: Effect of cortisone on survival of skin homografts in rabbits. Brit. Med. J. 1: 1157–1163, 1951.

(*100*) ———: Effect of locally applied cortisone acetate on survival of skin homografts in rabbits. Brit. Med. J. 2: 1049–1053, 1951.

(*101*) Maumenee, A. E.: The influence of donor-recipient sensitization on corneal grafts. Am. J. Ophth. 34: 142–152, 1951.

(*102*) Sugiura, K., Stock, C., Dobriner, K., and Rhoads, C. P.: The effect of cortisone and other steroids on experimental tumors. (Abstract.) Cancer Research 10: 244–245, 1950.

(*103*) Higgens, G., and Bennett, W.: The influence of cortisone acetate upon the growth of a transplanted rhabdomyosarcoma in C3H mice. J. Nat. Cancer Inst. 12: 851–860, 1952.

(*104*) Foley, E., and Silverstein, R.: Progressive growth of C3H mouse lymphosarcoma in CF_1 mice treated with cortisone acetate. Proc. Soc. Exper. Biol. & Med. 77: 713–714, 1951.

(*105*) Howes, E.: Cortisone and homologous transplantations of tumors. Yale J. Biol. & Med. 23: 454–461, 1951.

(*106*) Foley, E. J.: Effect of cortisone acetate on growth of strain specific tumors in alien strains of mice. Proc. Soc. Exper. Biol. & Med. 80: 669–671, 1952.

(*107*) Agosin, M., Christen, R., Badinez, O., Gasic, G., Neghme, A., Pizarro, O., and Jarpa, A.: Cortisone-induced metastases of adenocarcinoma in mice. Proc. Soc. Exper. Biol. & Med. 80: 128–131, 1952.

(*108*) Michael, M., Jr., and Whorton, C. M.: Delay of the early inflammatory response by cortisone. Proc. Soc. Exper. Biol. & Med. 76: 754–756, 1951.

(*109*) Spain, D., Molomut, N., and Haber, A.: Studies of the cortisone effects on the inflammatory response. I. Alterations of the histopathology of chemically induced inflammation. J. Lab. & Clin. Med. 39: 383–389, 1952.

(*110*) Menkin, V.: Cortisone and the mechanism of increased capillary permeability in inflammation. Federation Proc. 10: 91, 1951.

(*111*) Ebert, R. H.: Changes in connective tissue reaction induced by cortisone in vivo observations using the rabbit ear chamber technique. J. Clin. Invest. 30: 636–637, 1951.

(*112*) Asboe-Hansen, G.: Influence of ACTH and cortisone on dermal spread. Suppressed action of hyaluronidase due to connective-tissue changes. Acta Endocrinol. 9: 29–36, 1952.

(*113*) Layton, L.: Effect of cortisone upon chondroitin sulfate synthesis by animal tissues. Proc. Soc. Exper. Biol. & Med. 76: 596–598, 1951.

(*114*) ANDERSON, G., WIESEL, L., HILLMAN, R., and STUMPE, W.: Sulfhydryl inhibition as a mechanism in the effects of ACTH and cortisone. Proc. Soc. Exper. Biol. & Med. 76: 825–827, 1951.

(*115*) SYVERTON, J., WERDER, A., FRIEDMAN, J., ROTH, F., GRAHAM, A., and MIRA, O.: Cortisone and roentgen radiation in combination as synergistic agents for production of lethal infections. Proc. Soc. Exper. Biol. & Med. 80: 123–128, 1952.

(*116*) GARDNER, W. U.: Tumors in experimental animals receiving steroid hormones. *In* Endocrinology of Neoplastic Diseases. New York, Oxford Univ. Press, 1947, pp. 11–44.

(*117*) SELYE, H.: Experimental investigations concerning the role of the pituitary in tumorigenesis. *In* Endocrinology of Neoplastic Diseases. New York, Oxford Univ. Press, 1947, pp. 45–62.

(*118*) HALSTED, W. S.: Auto and isotransplantation in dogs of the parathyroid glandules. J. Exper. Med. 11: 175–199, 1909.

(*119*) DAMERON, J.: Homologous transplantation of fetal endocrine tissue to adult non-related host. Proc. Soc. Exper. Biol. & Med. 73: 343–345, 1950.

(*120*) TURNER, C.: Homoplastic transplantation of suprarenal glands of rat into the anterior chamber of the eye. Proc. Soc. Exper. Biol. & Med. 39: 133–135, 1938.

(*121*) TURNER, C., HAFFEN, R., and ST. AMANT, L.: Persistence of medullary tissue in homotransplanted adrenals. Proc. Soc. Exper. Biol. & Med. 41: 474–476, 1939.

(*122*) WOODRUFF, M. F., and WOODRUFF, H. G.: The transplantation of normal tissues: with special reference to auto- and homotransplants of thyroid and spleen in the anterior chamber of the eye, and subcutaneously, in guinea pigs. Phil. Trans. of the Roy. Soc., London, s.B. 234: 559–582, 1950.

PLATE 50

FIGURE 1.—Human epidermoid carcinoma, metastatic to neck. Original tumor, used for implantation in X-irradiated animals. Note necrotic area (*arrow*). × 140

FIGURE 2.—Same tumor as figure 1, 14 days after subcutaneous implantation as a suspension of minced tissue in the flank of an X-irradiated rat. There are no areas of necrosis. × 140

FIGURE 3.—Magnification of figure 2 showing numerous mitotic figures (*arrows*). × 550

FIGURE 4.—Same tumor as in figure 1, growing in another X-irradiated rat, 14 days after implantation. A blood vessel of the host (*arrow A*), entering the edge of the tumor, has adjacent mitotic figures (*arrows B*). There is no reactive tissue about the growth. × 660

FIGURE 5.—Human adenocarcinoma of the rectum, 14 days after subcutaneous implantation as a mince in the flank of an X-irradiated rat. Note characteristic morphology and many mitoses (*arrows*). × 280

FIGURE 6.—Another human adenocarcinoma of the rectum, 14 days after subcutaneous implantation as a mince in an X-irradiated rat. Arrows point to numerous mitoses. × 330

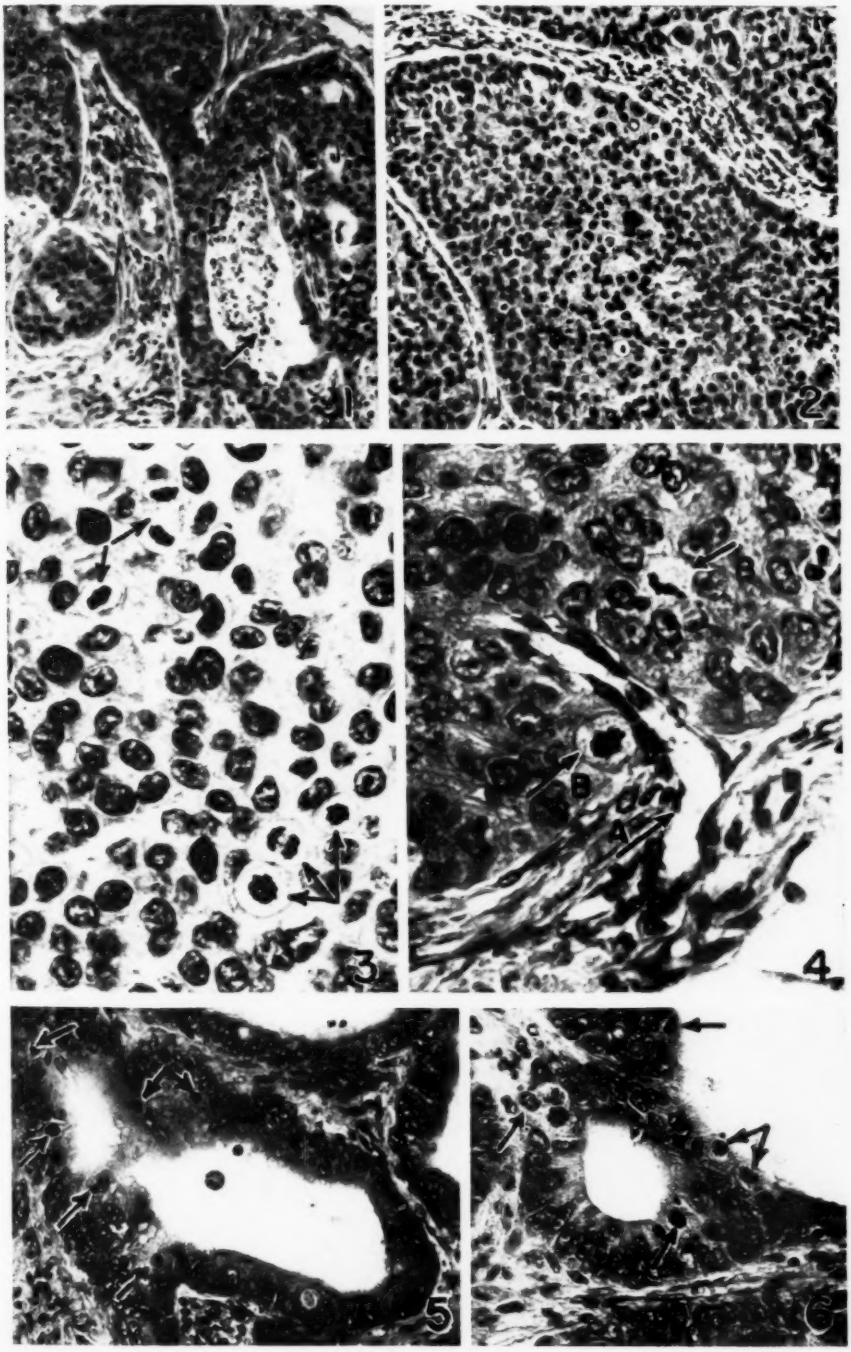


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PLATE 51

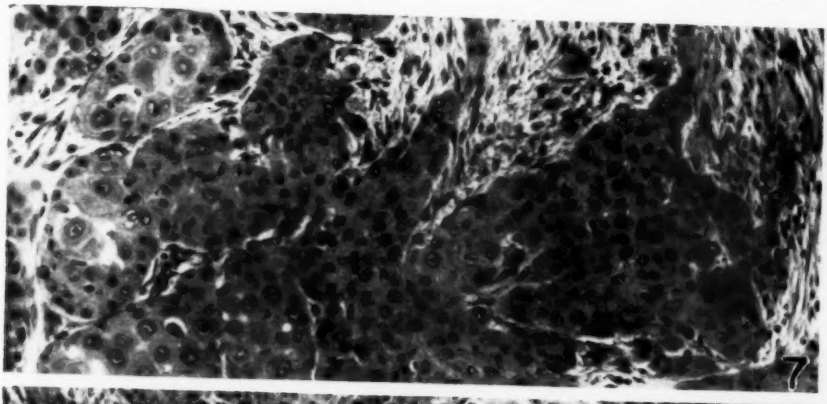
FIGURE 7.—Metastatic human epidermoid carcinoma from neck area. Original tumor. × 180

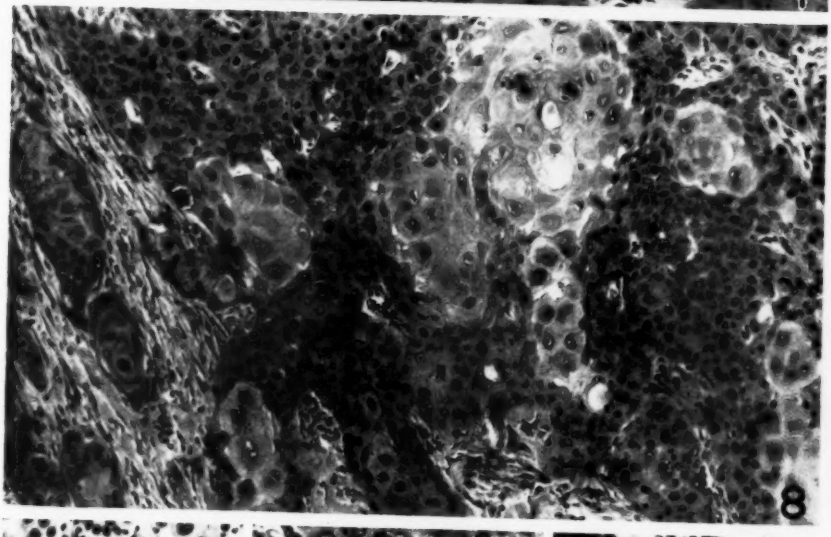
FIGURE 8.—Same tumor as in figure 7, 14 days after subcutaneous implantation as a suspension of minced tissue in an X-irradiated rat. Note extensive growth of the tumor and lack of reaction in the host stroma. × 140

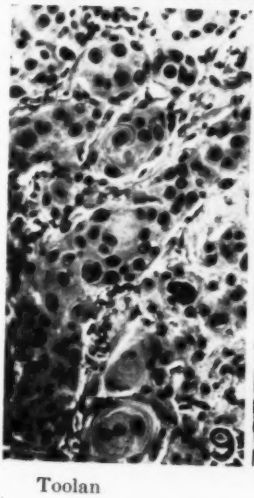
FIGURE 9.—Second generation of tumor (fig. 7) in X-irradiated rats. Transfer from growth of figure 8. × 180

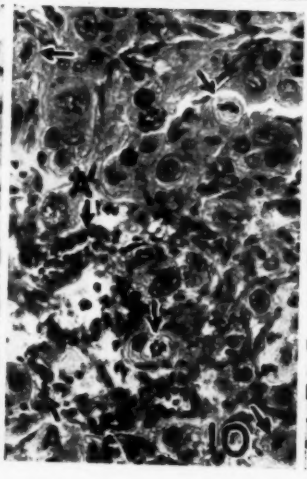
FIGURE 10.—Transfer of tumor (fig. 8) to allantoic membrane of chick—7 days' growth on membrane. Arrows labeled A point to blood vessels of chick with nucleated red cells. Plain arrows indicate mitoses. × 220

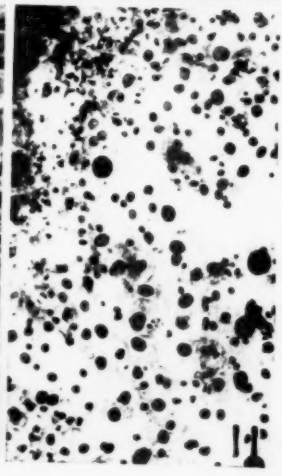
FIGURE 11.—Transfer of tumor (fig. 8) to tissue culture—17 days' growth. × 140











Toolan

Discussion: Methods of Conditioning the Host

Moderator: Dr. N. Kaliss, Roscoe B. Jackson Memorial Laboratory

DR. P. B. MEDAWAR, UNIVERSITY COLLEGE, LONDON, ENGLAND: May I add two footnotes to Dr. Toolan's able review of this most difficult problem of "conditioning the host"?

1) Dr. Billingham and I have repeated with great care the work by which Dr. Allen and his associates [1] purport to have demonstrated a "desensitization" of rabbits to skin homografts by repeated injections of extracts of donor skin. The work of these authors was incompletely controlled in the sense that *a*) they are not safeguarded from the claim that extracts of a rabbit's *own* skin into itself would bring about a comparable increase of survival time, and *b*) that the injection of a solution containing phenol as a preservative might prolong survival as the nonspecific consequence of a stress reaction. In general, Dr. Billingham and I are unable to confirm their claim that survival of homografts is prolonged by the injection of skin extracts, though it may be achieved, irregularly, by the injection of phenol solutions.

2) For completeness' sake, I should like to add to Dr. Toolan's account one naturally occurring example of the virtually complete "desensitization" to homografts of a normally reactive host: that of dizygotic twin cattle.[2,3] Homografts of skin may be freely interchanged between the great majority of dizygotic cattle twin pairs, even when they are of unlike sex and of conspicuously different genotypes. Presumably this is the outcome of the foetal vascular anastomosis which leads on the one hand to the freemartin condition in the females of pairs of unlike sex, and on the other hand to the uniformity of red-cell antigens which Owen [4] has shown to be the consequence of foetal cellular exchange. Indeed, dizygotic cattle twins are genetic chimeras at birth: they consist of (very unequal) mixtures of each other's cells! We prefer, incidentally, to speak of "actively acquired tolerance" rather than of "desensitization." This is not mere pedantry, for the latter term conceals an immunologic hypothesis that may be inapplicable, while the former is merely descriptive.

[1] ALLEN, H. L., WILLIAMS, R. D., LOVINGOOD, C. G., and ELLISON, E. H.: The effect of donor skin desensitization and ACTH on survival of skin homografts in rabbits. Ann. Surg. 135: 239-244, 1952.

[2] ANDERSON, D., BILLINGHAM, R. E., LAMPKIN, G. H., and MEDAWAR, P. B.: The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. Heredity 5: 379-397, 1951.

[3] BILLINGHAM, R. E., LAMPKIN, G. H., MEDAWAR, P. B., and WILLIAMS, H. L.: Tolerance to homografts, twin diagnosis, and the freemartin condition in cattle. Heredity 6: 201-212, 1952.

[4] OWEN, R. D.: Immunogenetic consequences of vascular anastomoses between bovine twins. Science 102: 400-401, 1945.

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